PHYSIOLOGICAL ZOÖLOGY

A Quarterly Journal of Zoölogical Research

CHARLES MANNING CHILD AND WARDER C. ALLEE
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Physiological Zoölogy

Vol. VIII JANUARY 1935 *No. 1*

THE HEAD FREQUENCY GRADIENT IN EUPLANARIA DOROTOCEPHALA[1]

(Twenty-nine figures)

C. M. CHILD AND Y. WATANABE
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VARIOUS lines of evidence indicate that in *Euplanaria* (*Planaria*) *dorotocephala* and certain other planarian species which undergo fission at a more or less definite post-oral level the bodies of the larger animals represent two or more zooids, which are physiologically—perhaps histologically to some extent—but not morphologically distinguishable (Child, 1910, 1911*c*, 1913, 1930). The anterior zooid includes the head and the body anterior to the level of fission which is a short distance posterior to the mouth but varies somewhat in position in different individuals and species. The body posterior to this level shows evidences of the presence of one or more zooids according to length, and a posterior growing region. Data given in earlier papers show that form and structure of the reconstituted planarian head and the frequency of development of a head differ in certain species with length of piece and level of body at which reconstitution occurs (Child, 1911*a*, 1912, 1916*a;* Behre, 1918; Buchanan, 1922; Šivickis, 1923; Abeloos, 1930). The various head-forms constitute a graded series representing different degrees of differential inhibition of head development, with the normal head at one extreme and the acephalic form at the other (Child, 1911*a*, 1921). For convenience the various members

[1] This investigation was supported in part by funds from a grant by the Rockefeller Foundation in aid of research in the biological sciences at the University of Chicago.

of this series have been grouped as normal, teratophthalmic, teratomorphic, anophthalmic heads and acephalic forms; and the frequencies of the various forms in lots consisting of pieces of a certain length and from a certain body-level of animals of the same length has been termed "head frequency." The references given above show that head frequency is related in a definite way to the zooids present in the bodies of individuals of a given length and that it also differs with physiological age, nutritive condition, and other physiological factors (Child, 1920) and can be altered and controlled by various external agents (Child, 1912, 1916*a;* Behre, 1918; Buchanan, 1922; Hinrichs, 1924); and more recent experiments not yet published have given additional data. In all these experiments essentially the same series of head-forms from normal to acephalic appears: in other words, the head-forms are not specific for physiological factors or for particular external agents. It has been shown by other experiments that susceptibility to KCN (Child, 1914*a*), oxygen consumption (Hyman, 1923), and CO_2 production (Robbins and Child, 1920) may be increased following section of the pieces from the original body and that such increase differs with length of piece and level of body. In pieces one-third or more of the body length of large animals it is slight or absent and becomes greater with decrease in length of piece. In the shorter pieces of equal length (one-sixth or less) from different levels, those from anterior regions of the anterior zooid and from the region of the posterior zooid or zooids show less increase in susceptibility and respiration than those from posterior levels of the anterior zooid. This increase in susceptibility and respiration is temporary and disappears in 12–24 hours or less. It has been regarded as evidence of stimulation of the piece by section (Child, 1914*a*, 1916*a;* Behre, 1918; Buchanan, 1922). The differences in head frequency in pieces of different length from different body-levels indicate that head frequency decreases as this effect of section increases. Moreover, other experiments have shown that the physiological factors which determine whether a head shall or shall not develop on a piece are so firmly established within 8–12 hours after section at a temperature of 20°–22° C. that head development is not prevented by greatly decreasing the length of the piece after this time, but may be more or less inhibited by the

decrease in length up to 24 hours (Child, 1914*b*). Evidently, determination whether a head shall develop occurs very soon after section; and at any particular level, the shorter the piece, the greater the stimulation and the lower the head frequency.

It was also found that head frequency could be either increased or decreased by the same toxic agent in the same concentration according to length of piece and level of body from which the piece was taken and length of period of exposure (Child, 1912, 1916*a;* Buchanan, 1922; Hinrichs, 1924). In pieces in which susceptibility and respiration show little or no increase following section, head frequency is decreased by ranges of concentration of the agents used which depress developmental activity: in pieces which show marked increase in susceptibility and respiration following section, head frequency may be increased, particularly with exposures ranging from a few hours to 2–3 days, by the same concentrations of the same agents. With caffein, however, exposures of 3–7 hours bring about changes in head frequency opposite in direction to those induced by depressing agents: with 20–46 hours' exposure the changes are like those induced by depressing agents (Hinrichs, 1924).

In the light of all these facts it was suggested in various papers (Child, 1916*a;* Behre, 1918; Buchanan, 1922; Hinrichs, 1924) that two antagonistic factors are concerned in determining head frequency in planarian pieces, particularly in the shorter pieces in which head development is more or less inhibited. These two factors were considered to be: first, the physiological condition of the cells directly concerned in head development; second, the physiological condition of other regions of the piece posterior to the region from which the head develops. Depressing or inhibiting action on the head-forming cells tends to decrease head frequency; such action on other parts of the pieces in which susceptibility and respiration are increased by section and head frequency is low tends to increase head frequency by inhibiting the factor which inhibits head development. The result in any particular case will depend on the relation of these two factors in the piece and the period of exposure to the agent.

The present paper is concerned with the results of more recent experiments which give more complete data on head frequency and

throw further light on the factors concerned in the reconstitutional determination and development of the planarian head. Some of the experiments described below have been used as laboratory experiments for groups of students during a number of years with highly satisfactory and uniform results.

THE HEAD-FORMS

The various head-forms between normal and acephalic constitute a graded series, the members of which represent different degrees of differential inhibition of head development. The five groups—normal, teratophthalmic, teratomorphic, anophthalmic, and acephalic—which constitute a convenient basis for distinction of the different degrees of inhibition, have been repeatedly described and figured (Child, 1916*a*, 1921; Šivickis, 1923, and other papers); and only their chief characteristics need be noted here.

The normal head is the form characteristic of the species in nature. In the genus *Euplanaria* it is triangular in form with two symmetrically placed eyespots and lateral cephalic lobes of auricles very slightly posterior to the level of the eyes.

The teratophthalmic head has the same outline as the normal, but the eyes are nearer the median line and the black pigment of the two is more or less connected. Forms included in the teratophthalmic group range from those in which the eyes are some distance from the median line, and connected only by a narrow strand of pigment, to forms in which the pigment of the two eyes is fused at the median line but the general form of the head is not altered.

In the teratomorphic head there is a single eye in the median line, the anterior margin of the head is rounded instead of pointed, the cephalic lobes arise anteriorly instead of laterally and show various degrees of approximation to the median line with a single median lobe as the extreme form.

In anophthalmic forms eyes are entirely absent, a single median cephalic lobe may be present anteriorly on the rounded head, or no cephalic lobe is present and the head is represented only by a tissue outgrowth.

Acephalic forms are those in which the new tissue merely heals and fills in the contracted concave cut end without outgrowth.

The various degrees of approximation to the median line and fusion of the eyes are paralleled by a similar approximation and fusion and decrease in size of the cephalic ganglia (Child and McKie, 1911). In anophthalmic forms the ganglia are represented only by a small median mass of cells, and in acephalic forms ganglia are entirely absent. It is evident that the inhibiting factor affects the median more than the lateral regions of the head (Child, 1921). In the lesser degrees of inhibition only the median region is absent, and adjoining regions are localized slightly nearer the median line than normally. With a greater degree of inhibition regions further lateral are absent, the eye locus becomes median, and with still further inhibition the locus of the cephalic lobes also becomes median. Then eyes and cephalic lobes do not appear, the ganglia develop only as a small median mass, and finally head development is completely inhibited. Asymmetries of size or position of the eyes appear not infrequently; many, if not all, of these appear to be due to incidental conditions such as differences in amount of parenchyma and entoderm exposed at the cut surface on the two sides, irregularities in closure of the wound, etc. Most of these asymmetries are temporary and become normal later.

Both physiological factors and external agents give rise to essentially the same series of head-forms in planarians, with some minor differences—for example, proportion of new and old tissue in the head, size of eyes, and degree of development of cephalic lobes. Moreover, the same mediolateral differential appears in modifications of development by internal and external factors in various other animals. In fishes, for example, the head-forms which appear in the experimental modification of development by chemical and physical agents and in hybridization show the same mediolateral differential in the reduction and failure of parts to develop.[2] In the experimental modification of amphibian development by external agents (Bellamy, 1919) a similar series of head-forms appears and certain amphibian hybrids show evidences of a similar differential inhibition in the head region (Montalenti, 1933). Again a similar mediolateral differential appears in the modification of

[2] See, for example, Newman, 1917, 1918; Child, 1921; Stockard, 1921; Hyman, 1921; Hinrichs, 1925; and literature cited by these authors.

larval development in the sea urchin by external agents (Child, 1916*b*). Evidently there is in all these cases a similar differential susceptibility to the inhibiting factors, whether they are physiological or external. The graded series of forms which appears in these modifications indicates a graded differential in physiological condition from the median to lateral regions as the basis of localization of the parts concerned.

The different frequencies of the head-forms in *Euplanaria* under different physiological and external conditions indicate different degrees or intensities of the inhibiting factors. The planarians, particularly the species *E. dorotocephala*, constitute the most favorable material available for the analysis of this type of developmental modification because it is possible to experiment with large numbers of pieces and to obtain numerical data on head frequency, and because the similarity of head-forms under different conditions and the relation of head-form to length of piece and level of body in animals of a given length and the relation to differences in physiological age (size) and nutritive condition, temperature, etc., make possible a considerable degree of standardization of the experimental material and therefore a more complete control of results than with various other forms.

HEAD FREQUENCY IN *Euplanaria dorotocephala* IN RELATION TO LENGTH AND BODY-LEVEL OF PIECE

The first data on head frequency showed definite and orderly differences according to length of piece and body-level, but the number of pieces recorded was small and the different head-forms were not as sharply distinguished as in later work. Head frequencies for pieces of a given length have been given in various papers (Child, 1912, 1916*a;* Behre, 1918; Buchanan, 1922; Hinrichs, 1924) in connection with experiments on the effect of various external agents and conditions on head frequency, and laboratory experiments on head frequency under various conditions have been repeatedly performed by students. All of these experiments show, first, that in normal environment head frequencies always show the same general relation to length of animal, length of piece in relation to length of animal and level of body, and second, that under various experi-

mental conditions the frequencies are altered in certain definite ways.

Head frequencies from more recent experiments are given in Table I and Figures 1–4. These represent larger numbers of pieces of different length and from different body-levels and show more

TABLE I

PERCENTAGE OF HEAD FREQUENCIES OF THIRDS, FOURTHS, SIXTHS, AND EIGHTHS OF ANIMALS 18 MM. IN LENGTH; 50 PIECES IN EACH LOT

Length and Level of Pieces	Normal	Teratophthalmic	Teratomorphic	Anophthalmic	Acephalic	Mean head frequencies
1/3–1	96	4	.....	.....	.....	99.2
1/3–2	94	6	.....	.....	.....	94.8
1/3–3	100	.....	.....	.....	.....	100.0
1/4–1	80	20	.....	.....	.....	96.0
1/4–2	26	54	12	4	4	78.8
1/4–3	100	.....	.....	.....	.....	100.0
1/4–4	100	.....	.....	.....	.....	100.0
1/6–1	48	48	4	.....	.....	88.8
1/6–2	12	26	6	16	40	50.8
1/6–3	6	16	6	10	62	38.8
1/6–4	28	26	14	8	24	65.2
1/6–5	54	36	2	2	6	79.2
1/6–6	98	2	.....	.....	.....	99.6
1/8–1	44	48	4	4	.....	86.4
1/8–2	.....	26	2	26	46	41.6
1/8–3	.....	6	8	14	72	29.6
1/8–4	.....	2	4	16	78	26.0
1/8–5	30	16	10	8	36	59.2
1/8–6	20	34	10	16	20	63.6
1/8–7	34	44	6	8	8	77.6
1/8–8	98	2	.....	.....	.....	99.6

clearly than the earlier data the relation of head frequency to length and level of piece. Since the experimental data concern pieces of equal length from different body-levels, but representing different fractions of postcephalic length, both length and level of piece are indicated numerically, length as a fraction of postcephalic length, level by a numeral, following the fraction, e.g., 1/3–1, 1/3–2, 1/3–3; 1/4–1 to 1/4–4; 1/8–1 to 1/8–8; etc., the level 1 being the most anterior level immediately posterior to the head.

Table I, from data obtained by the senior author, gives the numerical head frequencies in percentages for one-third, one-fourth, one-sixth, and one-eighth pieces of the postcephalic region of animals approximately 18 mm. in length from a single stock, all run at the same time at a temperature of 20°–22° C. These data are

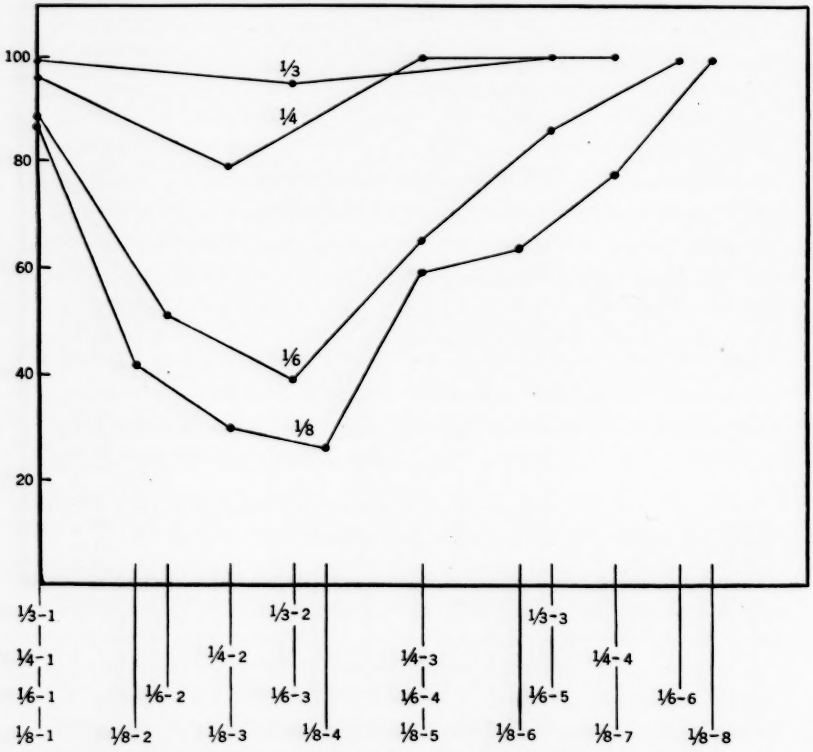


FIG. 1.—Head frequencies of thirds, fourths, sixths, and eighths of 18 mm. animals

graphed in Figure 1. The method used for graphing consists in assigning to the different head-forms arbitrary numerical values (normal, 100; teratophthalmic, 80; teratomorphic, 60; anophthalmic, 40; acephalic, 20[3]) and with these values determining the

[3] Under certain experimental conditions, death frequency also shows a differential distribution corresponding to the distribution of head frequency. For this reason the value 0 is reserved for deaths.

arithmetical mean percentage head frequency for each lot. The graph is plotted with these mean frequencies as ordinates and body-levels at which the heads develop as abscissae. Although this method involves use of the arbitrary values for the head-forms, it is employed for various reasons: first, it is desirable to distinguish the

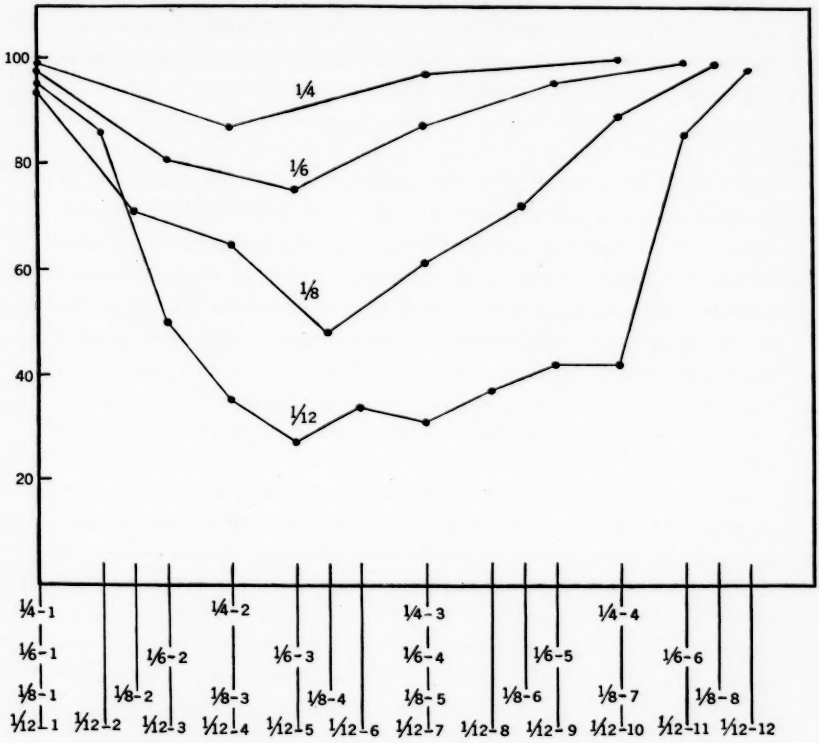


FIG. 2.—Head frequencies of fourths, sixths, eighths, and twelfths of 16–18 mm. animals.

five groups of head-forms because the differences in head frequency appear more clearly than with a smaller number of groups and because some of the groups, particularly teratomorphic heads, occur in small numbers; second, since the five groups form a graded series, this method gives an approximate mean and is much simpler than

other methods for this number of groups; third, the head-frequency curves obtained by this method serve comparative purposes and, as is evident from the data of Table 1, the essential points are not obscured by this method of plotting.

Figure 2, plotted in the same manner as Figure 1 from data obtained by the junior author, gives frequency curves for fourths, sixths, eighths, and twelfths of 16–18 mm. animals. The curves show in general the same differences in frequency with difference in length of piece and body-level as Figure 1, but the curves for pieces of the same lengths as those of Figure 1 are less steep and somewhat higher at their lowest points in Figure 2. The material of the two series came from different stocks collected at different times of year in different years and kept for different lengths of time in the laboratory after collecting, and the pieces were cut by different persons. In general, head frequency is somewhat lower in animals freshly collected than in those which have been well fed for several weeks in the laboratory. Difference in temperature may have played some part in the differences between Figures 1 and 2, since both series were run at room temperature and head-frequency curves are less steep and higher at their lowest points at higher than at lower temperatures.

Similar curves plotted year after year from the data of laboratory experiments performed by individual students and student groups all show the same general relations between head frequency, length of piece, and body-level, though the frequencies show some variation with different stocks of animals, nutritive condition, care in preparation of pieces, and temperature. Two series of such curves are given in Figure 3. In this figure the curves drawn in unbroken lines are plotted from the combined data of experiments performed at the same time by a group of nine students with twenty pieces per student in each lot. The curves drawn in broken lines are plotted from a similar series of data of another year and another group of five students, again with 20 pieces per student in each lot. In both of these series the data of individual students differ but little from the combined data. In these two student-series the head frequency is in general slightly higher and the decrease from anterior to pos-

terior region of the first zooid somewhat less than in pieces of the same length in Figure 1 and somewhat greater than in Figure 2, except in the one-fourth pieces. Figures 1, 2, and 3 represent data obtained by sixteen different persons from animals of approximately

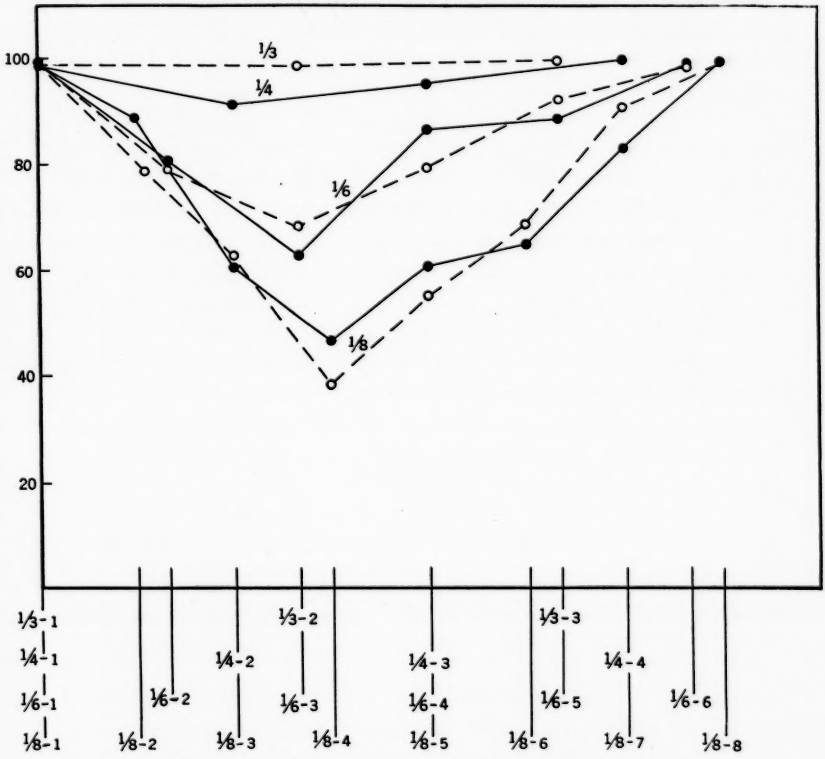


FIG. 3.—Two series showing head frequencies of thirds, sixths, and eighths and fourths, sixths, and eighths of 16–18 mm. animals from student data.

the same length but from different stocks and during different years. They record results from more than 6,000 pieces, and all show that head frequency decreases from anterior to posterior regions of the anterior zooid and increases again in the posterior zooid region, and that these changes in frequency increase as length of piece decreases.

Figure 4, also from data obtained by the junior author, gives head-frequency curves for pieces of animals 8–10mm. in length—that is, about half the length of the animals of Figures 1–3. Figures 2 and 4 are from large and small animals of the same stock, and comparison shows that in pieces representing the same fraction of

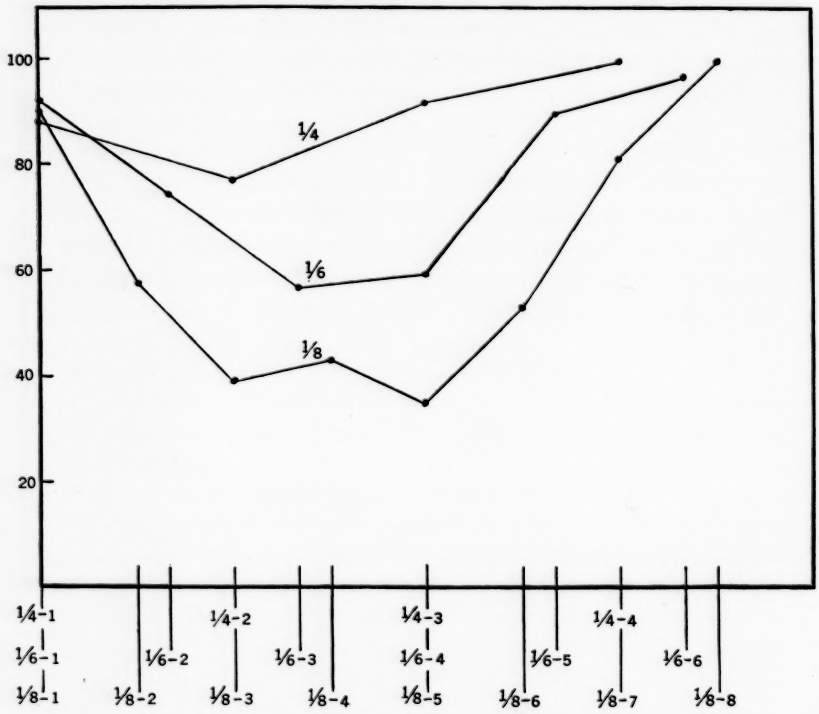


FIG. 4.—Head frequencies of fourths, sixths, and eighths of 8–10 mm. animals

body length the change in head frequency at different levels is greater in the small than in the large animals. Repeated experiments have shown that such difference between large and small animals is characteristic when experiments are performed with animals of the same stock and under similar conditions, but does not always appear in data obtained at different times from animals

of different stocks. Although the smaller animals are physiologically younger than the larger, the difference does not mean that young animals are less capable of reconstitution than old but results merely from the fact that pieces representing a given fraction of body length are only half as long in the small, as in the large, animals. Some data concerning this difference in head frequency in relation to size of animal were given in an earlier paper (Child, 1920).

It is evident from all the data that an increasing degree of differential inhibition of head development occurs as length of piece decreases, except at the anterior level, where the differences are slight between one-third and one-twelfth pieces, and at the extreme posterior end of the body, where they are practically absent. Moreover, the degree of differential inhibition increases from the anterior to the posterior region of the anterior zooid and decreases in the posterior zooid region.

One other point of interest which the mean head frequencies do not show appears in the data of Table I. In the pieces 1/6–4 and the pieces 1/8–5 and 1/8–6 the distribution of the frequencies differs from that in the other one-sixth and one-eighth pieces in that the frequencies of normal heads and acephalic forms are almost equal and the intermediate head-forms are also rather numerous. In pieces from other body-levels the highest frequencies occur either toward the normal or toward the acephalic end of the series; that is, either normal heads are distinctly more frequent than acephalic forms or the reverse. The 1/6–4 and the 1/8–5 and 1/8–6 pieces show a greater degree of heterogeneity than other body-levels in the distribution of head frequencies. This heterogeneity in pieces from these levels is a characteristic feature of such series and is interpreted as due to the presence of the anterior end of the second zooid in the region from which these pieces are taken. Since the level of the second zooid differs somewhat in different individuals, and since corresponding pieces from different individuals are not exactly equal in length or from exactly the same region, some of the 1/6–4 and the 1/8–5 and 1/8–6 pieces may represent only the posterior region of the first zooid, and others may include the anterior end of the second zooid. In the first case, the head frequency will be low and acephalic forms will predominate. In the second case,

head frequency will be high and normal, or normal and teratophthalmic heads will predominate. Consequently in a lot in which both sorts of pieces are present in numbers determined by chance there will be a wider distribution of the frequencies with more nearly equal frequencies at the two ends of the series than in other lots from levels where the physiological conditions are more nearly uniform. This interpretation of the observed facts is in agreement with other lines of evidence indicating the presence of a posterior zooid region differing physiologically from the region immediately anterior to it. The occurrence of fission at a more or less definite postoral level with some degree of independent muscular activity in the region posterior to this level preceding fission (Child, 1910), the difference in susceptibility of the regions anterior and posterior to this level (Child, 1913, 1930), the distribution of head-forms, and the increase in head frequency at this level—all agree in indicating that there is here a more or less sharply defined boundary between regions which differ from each other physiologically in some way. The only possible conclusion in the light of all the facts is that the posterior region of the body of the larger individuals of this and various other planarian species which undergo fission at a definite level represents physiologically one or more zooids. In other words, this part of the body has become more or less physiologically isolated (Child, 1929) as increase in length has occurred; and consequently some degree of reorganization into a new individual, perhaps involving only or chiefly the nervous system, has taken place, but this reorganization does not proceed far until physical isolation by fission is complete.

HEAD FREQUENCY IN VERY SHORT ANTERIOR AND POSTERIOR PIECES

Table I and Figures 1–4 show that in pieces from the extreme anterior and posterior regions of the body the decrease in head frequency with decrease in length of piece is very slight or absent, even in one-eighth and one-twelfth pieces. Table II gives head frequencies of still shorter pieces from these regions. In these experiments pieces approximately 1 mm. in length were cut from the anterior (*A*, *B*, Fig. 5) and extreme posterior regions (*X*, *Y*, *Z*, Fig.

5) of animals 18–20 mm. in length. Since the two anterior regions, *A* and *B*, are known to give practically 100 per cent head frequency in the longer pieces, they are not separated in the table; but data

TABLE II

PERCENTAGE OF HEAD FREQUENCIES IN VERY SHORT ANTERIOR AND POSTERIOR PIECES

Region of Body (Fig. 5)	Number of Pieces	Normal	Teratophthalmic	Teratomorphic	Anophthalmic	Acephalic	Dead	Means of Living
A and *B*	50	16	42.0	2.0	6.0	22.0	12	65.5
*X**.....	200	41	25.5	9.0	12.0	9.5	3	75.8
*Y**.....	200	58	26.0	6.0	6.5	2.0	1	86.0
*Z**.....	200	77	16.0	3.5	1.5	1.5	2	94.0

* Data for the *X*, *Y*, and *Z* pieces were obtained by Mr. George Lee, graduate student.

for the three posterior pieces, *X*, *Y*, and *Z*, are given separately. The anterior pieces show marked inhibition of head development, 22 per cent being acephalic and the mean frequency being only 65.5, as compared with frequencies ranging from 86 to 96 for anterior eighths and twelfths in Figures 1–3. In the posterior pieces, *X*, *Y*, and *Z*, Table II, the frequencies are higher and increase as the level becomes more posterior, being highest, 94, in the extreme posterior piece which has no posterior cut surface. Of the *X* pieces 56 per cent are more or less inhibited and 9.5 per cent acephalic; of the *Y* pieces 41 per cent and of the *Z* pieces only 21 per cent are inhibited, and very few of these are acephalic. It is evident from these data, first, that even in extreme anterior and posterior regions inhibition of head development occurs when the pieces are cut short enough; second, that decrease in length of piece is less effective in inhibiting head development in the extreme posterior, than in the anterior, region; and third, that in the terminal posterior piece which has no cut surface but is considerably less in volume than the other pieces length has less effect on head frequency than in any other region of the body.

FIG. 5.—Body-levels of pieces *A*, *B*, *X*, *Y*, and *Z* of Table II.

THE EFFECT ON HEAD DEVELOPMENT OF DELAY OF ANTERIOR OR POSTERIOR SECTION

The fact that in pieces with cut surface at both ends the development of the head is increasingly inhibited as length of piece decreases suggests the possibility that conditions resulting from the presence of the posterior cut surface play some part in the inhibition. Either such conditions may conceivably be direct effects of the injury, such as stimulation of the nervous system or production of toxic substances, or they may be the developmental activities at the posterior end which begin soon after section. These possibilities have suggested the experiment of making anterior or posterior transverse sections at different times. Experiments of this sort have been repeatedly performed by one of us and by students with definite results which throw some further light on the problem of physiological inhibition of head development.

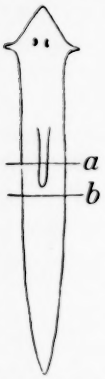


FIG. 6.—Body-levels of delayed anterior (*a*) and posterior (*b*) section.

Anterior later than posterior section.—For this experiment animals of approximately the same length, 16–18 mm., which have not recently undergone fission and therefore have well-developed posterior zooid regions of approximately equal length, are selected. Since head development is most inhibited in short pieces from the posterior region of the first zooid, we may expect that any effect of conditions arising from the posterior cut end on head development at a delayed anterior cut end will appear most clearly in such pieces. Procedure is as follows: For controls, pieces *ab*, Figure 6, one eighth to one-tenth of the postcephalic length are cut. These pieces develop almost entirely or entirely acephalic forms. Since it is known that pieces one-third or more of the body length with anterior ends at the level *a*, Figure 6, develop almost or quite 100 per cent normal heads, a second control lot of long pieces with anterior ends at the same level as those of the short pieces, *ab*, is unnecessary, except for the purpose of making the situation clear to students. For the experiment a large number, 100 or more, depending on the number to be used in each experimental lot, of pieces consisting of

the head and body anterior to the level *b*, Figure 6, are prepared in lots of 20 or more. From successive lots of these pieces, for example, of 20 pieces each, the pieces *ab*, Figure 6, are obtained by making the section at *a* at a different interval following the section at *b* in each lot. These lots of *ab* pieces are allowed to develop and their head frequencies determined. In these pieces head development at *a* begins at different times after the cut surface at *b* is es-

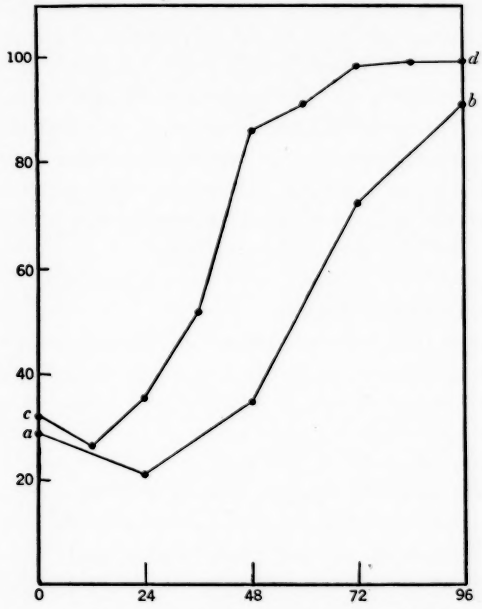


FIG. 7.—Two series of head frequencies with delayed anterior section

tablished. In Figure 7 the mean percentage head frequencies from two series of such experiments are graphed. The curve *ab* gives the frequencies of lots of 50 pieces each with anterior end established 0, 24, 48, and 96 hours later than the posterior end. At 24 hours the frequency is lower than in the control (0 hours), but increases, from this time to 96 hours, though it does not attain 100 per cent even at that time. The curve *cd* is plotted from experiments performed by six students at the same time with control and experi-

mental lots of 20 pieces each, a total of more than 600 pieces. Different members of the group used different time-intervals, so that from the data of the group as a whole frequencies for each 12 hours of delay of the anterior section were available. In this student-series head frequency increases more rapidly than in the other series. This difference may be due to various factors, condition of material, temperature, etc., but is probably due chiefly to somewhat greater length of pieces in the student-series. It is very generally the case that students unaccustomed to the preparation of pieces representing a small fraction of body length make the pieces somewhat too long. This experiment has been repeated by student groups from year to year with essentially the same result, but with differences in steepness of the curve and in the time of attainment of 100 per cent. In pieces of the same length, this effect of delaying the anterior section decreases as level of piece becomes more anterior, and in the posterior zooid region it is also less than in the experiments cited. In pieces with anterior ends at a given level, it decreases with increase in length of piece.

Posterior later than anterior section.—In this experiment the same body-level may be used as in the preceding, and the procedure is similar. The control in which anterior and posterior section are made at the same time consists again of the pieces *ab*, Figure 6, one-eighth to one-tenth of the postcephalic length; these pieces develop almost or quite 100 per cent of acephalic forms. The experimental pieces consist of the body posterior to the level *a*, Figure 6; such pieces develop 100 per cent normal heads. From the anterior ends of successive lots of these pieces the pieces *ab* are cut at different intervals by making the section at *b*, allowed to develop, and their head frequencies determined. In this experiment the increase in head frequency with delay of posterior section is much more rapid than in the preceding: with 8 hours' delay of posterior section all pieces usually develop heads of some kind, and with 12–24 hours' delay all, or almost all, are normal. Figure 8 shows the mean frequencies of three series of experiments. Curves *ab* and *cd* are from experiments by the senior author with lots of 25 pieces each in each series. In *ab* the posterior cut was delayed 6 and 12 hours; in *cd*, 4, 8, 18, and 24 hours. The level of body used

was slightly farther anterior in the second series; and as the graph shows, the frequencies are slightly higher in this (*cd*) than in the first (*ab*). The curve *ef*, Figure 8, is plotted from the data obtained by a student group of seven individuals with lots of 20 pieces each per student. Different members of the group used different periods of delay. The curve plotted from all the data shows irregularities

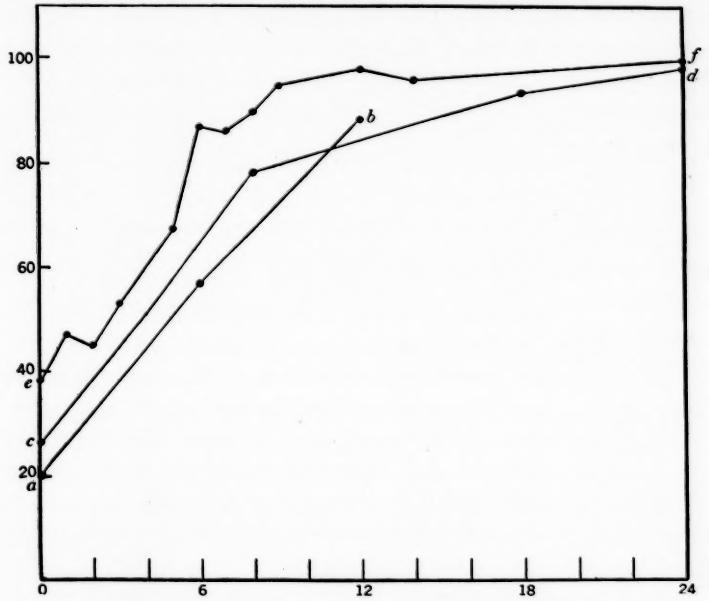


FIG. 8.—Three series of head frequencies with delayed posterior section

but is obviously similar to *ab* and *cd*. This experiment also has been repeated year after year by students, always with essentially the same result, that is, that inhibition of head development by the delayed posterior cut decreases rapidly up to 8–12 hours of delay and with 24 hours' delay is almost or entirely absent. Numerical data of two such experiments were given in an earlier paper, as evidence on the question of the time of head determination. As in the case of delayed anterior section, the inhibiting effect of the delayed posterior section in pieces of given length is less the more

anterior the level, is also less in pieces from the posterior zooid region than in the experiments cited, and decreases as length of piece with anterior end at a given level increases.

SECTION OF ACEPHALIC AND OTHER FORMS WITH INHIBITED HEADS

Unless extremely short, acephalic forms usually develop more new tissue at the posterior end than do forms with heads. The posterior half, more or less, of such forms represents a posterior zooid; and these forms often undergo fission near the middle by longitudinal contraction of anterior and posterior regions in opposite directions while attached to the substratum. If the anterior end of an acephalic piece is removed at the level x (Fig. 9) or posterior to this level, a normal head almost always develops on the remaining piece, AB; but if the anterior end is removed at the level x and the remainder of the piece separated into two pieces by section at y, the piece A almost invariably remains acephalic and a normal head develops on the posterior piece B. Table III gives percentage head frequencies observed after both of these operations. Many repetitions of these experiments by students and others have always given similar results, a very high normal frequency in pieces AB and B, often 100 per cent, and a very low frequency, often 100 per cent acephalic, in the A pieces. The anterior ends of the pieces AB and A represent approximately the same level of the acephalic piece, but the posterior cut surface is absent in pieces AB and B and present in A. Apparently conditions resulting from the presence

FIG. 9.—Outline of acephalic form indicating levels of section for pieces AB, A, and B, Table III.

TABLE III

HEAD FREQUENCIES AFTER SECTION OF ACEPHALIC FORMS

Piece	Number of Pieces	Normal	Teratophthalmic	Teratomorphic	Anophthalmic	Acephalic	Means
AB.....	50	92	2	2	2	2	96.0
A.....	50	.....	.....	.....	2	98	20.4
B.....	50	94	4	.....	.....	2	97.6

of the posterior cut surface or the developmental activities which occur there inhibit head development in the piece *A*, while in *AB* and *B* there is no posterior cut surface and head development is not inhibited. When fission occurs in acephalic pieces, head frequency is 100 per cent in the posterior fission pieces.

In pieces which develop teratophthalmic, teratomorphic, or anophthalmic heads, removal of these heads without a posterior section is followed by development of normal, instead of inhibited, head-forms; but if a posterior section is made when the head is removed, head development may again be inhibited, but the degree of inhibition may be more or less than in the first development, according to length of piece. Since the individual resulting from the first development of the piece is smaller than the original animal from which the piece was taken, pieces of given length from the reconstituted body represent a larger fraction of its total length and develop a higher head frequency than pieces of the same length from the original animal.

THE EFFECT ON HEAD DEVELOPMENT OF POSTERIOR HALF-SECTION

Since the preceding experiments indicate that conditions associated with the posterior section are concerned in inhibiting head development, the question of the effect of section halfway across the body is of interest. Will such section inhibit the half of the head, anterior to it, or will it inhibit the whole head to a greater or less degree? Three series of experiments have been performed by the junior author for evidence on this point. The first of these consisted in making a half-section a short distance posterior to the anterior cut end of the piece. In a second series a similar half-section was made and was reopened after 24 hours and every 12 hours thereafter during the next 4 days. Since these procedures did not inhibit head development to any great degree, a third series was run, in which a piece half the width of the body was removed posterior to the half-section.

Single and reopened posterior half-section without removal of tissue.—In these series pieces *A* (Fig. 10) with anterior ends just posterior to the head and *B* with anterior ends just posterior to the

mouth from 16–18 mm. animals were used. In each piece a transverse section was made from one side of the body to the median line 1–1.5 mm. posterior to the anterior cut end of the piece, that is, one-twelfth or less of the body length, as indicated in Figure 10.

FIG. 10.—Posterior half-section at anterior and postoral levels (Table IV).

In one series of pieces from each of the two levels this half-section was made only once and was allowed to heal: in another series from each level it was reopened repeatedly, as noted above. The pieces were examined daily during the first week of head development; and the asymmetry or symmetry of new tissue, head-form, and eyes was recorded. The two body-levels used in this experiment are the levels of highest and lowest head frequency in the anterior zooid (see Table I and Figs. 1–4), and the pieces are each approximately half the body length—that is, long enough to give 100 per cent normal heads in the absence of the half-section. The results of the experiments are given in Table IV. The last three columns of the table give the percentages of different head-forms: the column headed "symmetrical" includes cases in which head and eyes are symmetrical in all respects; the column "asymmetrical," cases in which asymmetry of head or eyes or both occurs in consequence of retardation of development on the side of the half-section as compared with the other side; the column "reversed," cases of the reverse asymmetry, that is, of retardation on the side without posterior half-section. The table shows that both with single and reopened half-section, 95.3 and 96.3 per cent of the heads which develop at the anterior level are entirely symmetrical. They are, in fact, completely normal heads without any visible inhibition of development either on the side of the half-section or of the head as a whole. At the postoral level, however, development is retarded on the side of the half-section in 19 per cent after single, and in 43.5 per cent after reopened, half-section. Reversed asymmetry occurs in less than 3 per cent of each lot and is undoubtedly due to incidental factors which have no relation to the half-section.

The head frequencies after complete transverse section in pieces

of approximately the same length as the distance between the anterior end and the half-section are almost 100 per cent normal at the anterior level (1/12–1, Fig. 2) and about 90 per cent acephalic at the postoral level (1/12–6, 1/12–7, Fig. 2). With the half-section the retarding effect is much greater at the postoral, than at the anterior, level, but very much less than with complete transverse section. None of the differentially inhibited head-forms—teratophthalmic, teratomorphic, anophthalmic—and no acephalic forms occurred among the 427 pieces of Table IV. The only effect was a retardation of development on the side of the half-section making the heads temporarily asymmetrical. Figures 13–27 drawn from

TABLE IV

HEAD DEVELOPMENT AFTER SINGLE AND REOPENED POSTERIOR HALF-SECTION WITHOUT REMOVAL OF TISSUE

Operation	Level of Head Development	Numbers of Pieces	Head Forms (Percentages)		
			Symmetrical	Asymmetrical	Reversed
Single.....	anterior	106	95.3	3.8	0.9
	postoral	105	78.1	19.0	2.9
Reopened....	anterior	108	96.3	3.7	.....
	postoral	108	53.7	43.5	2.8

the following series of experiments also serve to show the head-forms of the present series. These asymmetrical heads become completely normal in later development. At the anterior level the frequency of asymmetrical heads was no greater after reopened than after single half-section, but at the postoral level reopening of the half-section more than doubled the frequency of asymmetrical forms. The data show that posterior half-section even at a short distance from the level of head development, whether single or reopened, does not appreciably inhibit head development at the anterior level but at the posterior level does temporarily retard development on the side of the half-section, and at this level retardation is more frequent when the half-section is reopened. The differentially inhibited head-forms of short pieces with complete posterior section in which there is progressive elimination of parts

of the head from median to lateral regions do not appear at all after posterior half-section.

Posterior half-section with removal of tissue.—The operation in these experiments is indicated in Figure 11. Since even complete transverse section is less effective in inhibiting head development at the anterior than at the postoral level, and half-section alone has no effect at the anterior level, only the level immediately posterior to the mouth where posterior section is found to be most effective was used in these experiments. As in the preceding series the posterior half-section was made one-twelfth or less of the body length from the anterior cut surface, and a piece half the width of the body and 3.5–4 mm. long was removed posterior to the half-section. Results are given in Table V. The data for the three lots of pieces prepared are given separately to show the range of variation in frequencies in the different lots. Like the preceding series, this shows a retardation of development on the side of the half-section.

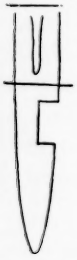


FIG. 11.—Posterior half-section with removal of piece (Table V).

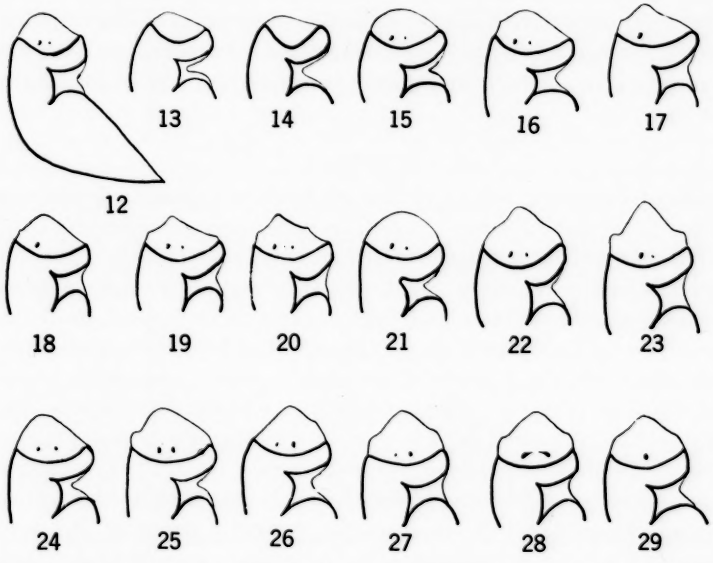
TABLE V

HEAD DEVELOPMENT AFTER POSTERIOR HALF-SECTION WITH REMOVAL OF TISSUE

LOTS	NUMBERS OF PIECES	HEAD FORMS (PERCENTAGES)		
		Symmetrical	Asymmetrical	Reversed
I	35	8.6	79.9	11.5
II	27	18.5	81.4	.....
III	38	13.2	81.5	5.3
Averages	.....	13	81	6

A total of 81 per cent of asymmetrical heads appears in the table, and the percentage is almost the same in each of the three lots. This is about double the percentage of asymmetrical heads in the preceding series. Apparently the removal of the piece in connection

with the posterior half-section increases its effectiveness. The frequency of reversed asymmetry is also higher in Table V than in Table IV but differs in the three lots. The variations in head-form are shown in Figures 12–29. In consequence of the contraction of the cut surfaces the pieces have the form indicated in Figure 12; but as new tissue develops in place of that removed, gradual straightening occurs. Asymmetry of the new tissue before eyes appear is



FIGS. 12–19.—Head-forms which develop after posterior half-section

shown in Figures 13 and 14. Figures 15–29 show head-forms 5–6 days after section. In Figures 15–20 asymmetry appears in shape of head, position of median plane, and development of eyes. In Figures 21–23 the head-form is symmetrical, the eyes asymmetrical. Figures 24 and 25 are cases of asymmetrical head-form with symmetrical eyes. Figures 26 and 27 are two of the six cases of reversed asymmetry. Figures 28 (teratophthalmic) and 29 (tera-

tophthalmic or perhaps slightly teratomorphic) show the only cases among the 100 pieces of head-forms characteristic of completely isolated short pieces. All of the asymmetrical forms tend to become symmetrical as development progresses.

It is evident from the data that even removal of a piece from one side of the body a short distance posterior to the level of head development does not bring about the inhibition of head development that occurs in short pieces with complete posterior section, but merely retards development on the side of the injury. The question of the physiological basis of this difference in effect on head development of complete and posterior half-section will be considered below.

DISCUSSION

Head frequency and inhibition of head development.—It appears from Table I and Figures 1–4 that in *Euplanaria dorotocephala* head frequency varies in a definite and orderly manner with length of piece and level of body. In the anterior region decrease in head frequency with decrease in length of piece is very slight until the pieces become extremely short. The effect of length increases from anterior to posterior levels of the anterior zooid—that is, in pieces of equal length from different levels, the shorter the pieces, the greater the decrease in head frequency. In the posterior zooid region the effect of length of piece decreases again toward the posterior end, and at the extreme posterior end is less than in any other region. When short pieces from the posterior region of the first zooid are completely isolated, head frequency is low, as Table I and Figures 1–4 show; but when either anterior or posterior section is delayed, head frequency increases with increase in the period of delay. In pieces which give a high acephalic frequency when completely isolated, head frequency increases with increasing delay of the posterior section up to 24 hours, when it becomes 100 per cent normal (Fig. 8). In similar pieces delay of anterior section increases head frequency up to 4 days, when it becomes almost, or quite, 100 per cent (Fig. 7). These experiments indicate that conditions associated with, or resulting from, the presence of the posterior cut surface are the physiological factors which inhibit head develop-

ment in this species. The continuation of the inhibiting effect during 4 days or more of delay of posterior section indicates further that not only the cut surface itself but also conditions associated with the earlier stages of development of the posterior end inhibit head development to some extent. The second operations on acephalic pieces point to the same conclusion. The pieces with fully developed posterior ends, even though only half the length of the original acephalic piece, develop almost 100 per cent normal heads.

In short, all the evidence seems to show, first, that the posterior injury or the activities resulting from it are the physiological factors which inhibit head development; second, that at a given body-level the effectiveness of the inhibiting factor increases as length of piece (that is, the distance between the level of head development and the posterior cut surface) decreases; third, that the length of piece (that is, the distance over which the inhibiting factor is effective) increases from anterior to posterior levels of the anterior zooid and decreases again in the posterior zooid region. The presence of a factor inhibiting head development was recognized in earlier papers, and it was regarded as resulting in some way from the increased physiological activity brought about by section in the old tissue of the piece posterior to the region directly concerned in head development. Since the evidence showed that head frequency decreased as this stimulation increased, it was inferred that when this effect of section was sufficiently intense it inhibited head development to a greater or less degree. These and other experiments on planarian reconstitution led to the general conclusion that the head region is primarily a self-determining region and that the physiological action of other parts of the piece on head development is inhibitory, not determinative—in other words, that the head develops in spite of the rest of the piece, and its development may be more or less inhibited or completely prevented by physiological correlative factors originating in the piece posterior to the level of head development. The data of the present paper do not alter this conclusion but permit a further analysis of the factors concerned in determining presence or absence and character of the head.

The nature of the inhibiting factor and the conditions determining head development.—The experiments on delay of anterior section

indicate that the factor inhibiting head development decreases in effectiveness from the time of section—at first rapidly, then more slowly—but is still evident after 3 or 4 days, that is, after healing and some growth of new tissue have occurred at the posterior end. The general stimulation resulting from section, as indicated by increased respiration (Robbins and Child, 1920; Hyman, 1923) and susceptibility of pieces (Child, 1914*a*) disappears in a much shorter time. Apparently this general stimulation is not itself the inhibiting factor, though the two are undoubtedly associated as results of section. Moreover, the experiments on delay of posterior section indicate that the inhibiting factor acts on the earliest stages of head development, for a delay of 8 hours in making the posterior section is sufficient to permit all pieces to develop heads, and with a delay of 24 hours all heads are normal. Apparently the inhibiting factor prevents or decreases the activation, proliferation, and growth of the cells at the anterior end which give rise to the head. The persistence of the inhibiting factor after healing of the posterior cut surface and the fact that this factor is itself inhibited by exposure of the pieces for a day or two to KCN (Child, 1916*a*) or anesthetics (Buchanan, 1922) suggest that it may be nervous in character rather than an inhibiting substance produced at the posterior cut.

On the basis of the evidence at hand the hypothesis is advanced that section of the longitudinal nerve cords by the posterior cut and secondarily the cellular activity at the posterior cut end of the piece determine a persistent excitation or change in condition in the nerve cords which is effective within a certain distance anteriorly in the piece. If the piece is short enough, this factor may inhibit to a greater or less degree the activation of the cells which give rise to the head. In the anterior region of the body this inhibiting factor is effective only over a very short distance; that is, inhibition of head development occurs only in very short pieces. In the posterior region of the anterior zooid it is effective over a considerably greater length of piece, and in the posterior zooid region its range of effectiveness is again less and decreases posteriorly. These differences at different body-levels must be due either to a gradient in physiological condition of the cells concerned in head development which makes necessary a different degree or intensity of the inhibiting

factor at different levels for inhibition or prevention of head development or to a difference in steepness of the decrement in the inhibiting factor at different levels. That there is a gradient in physiological condition, at least in the anterior zooid, is indicated by the data on differential susceptibility (Child, 1913), oxygen consumption (Hyman, 1923), CO_2 production (Robbins and Child, 1920), and by the differences in rate of eye development at different levels (Watanabe, 1935). If this gradient determines a difference in rate of activation and dedifferentiation of the head-forming cells following section, it seems probable that where activation and dedifferentiation are more rapid a greater inhibiting action is necessary to prevent their occurrence. According to this suggestion, the inhibiting factor may be essentially the same at all levels and the difference in head frequency is determined by a graded difference in physiological condition of the cells which determines a difference in susceptibility to the inhibiting factor. Moreover, according to the data on head frequency in the shorter pieces, the susceptibility to the factor inhibiting head development must increase from anterior to posterior levels of the anterior zooid and decrease again in the posterior zooid region. As already noted, the data on susceptibility, respiration, and head frequency all agree in indicating the existence in *Euplanaria dorotocephala* of a physiological gradient involving respiration. Even Parker's data on CO_2 production in *E. novangliae* (*Planaria maculata*), which he regards as evidence against the existence of such a gradient, give evidence for its existence. (Parker, 1929). In his Table IV, which gives CO_2 production for one-sixth pieces, including the second to the fifth one-sixth, a slight gradient similar to that in *E. dorotocephala* appears. His figures show a slight decrease in CO_2 production from the second to the third one-sixth, a slight increase from the third to the fourth one-sixth, and a greater increase from the fourth to the fifth one-sixth; but whether the differences are significant cannot be determined from the data given. His data on anterior and posterior halves (Parker, 1929, Table III) do not, and cannot be expected to, show a gradient because *E. novangliae*, like *E. dorotocephala*, possesses a posterior zooid region and at certain seasons of the year the terminal reproductive organs are also present in the posterior

half. Parker does not state whether reproductive organs are present or not in his material. In any case, his data do not conflict with the findings on *E. dorotocephala*, and his conclusion is apparently due to his belief that the posterior half of the body should show a lower CO_2 production than the anterior half if a gradient were present. In short, all the evidence at hand agrees in indicating the presence of a physiological gradient with decrease in activity from anterior to posterior levels of the anterior zooid and increase posteriorly in the posterior zooid region. As regards the latter region, various lines of evidence indicate that the increase toward the posterior end occurs in two or three steps in the longer animals; that is, that two or three posterior zooids are present (Child, 1910, 1911*c*, 1913, 1930).

The other suggestion of a difference in steepness of decrement in the inhibiting factor at different levels to account for difference in head frequency in pieces of the same length from different levels also involves the assumption of a graded physiological differential of some sort which determines the steepness of decrement. For example, it is possible that the normal anteroposterior dominance is less easily overcome and reversed in regions near the head and in the posterior zooids than elsewhere and that this differential corresponds to the head frequency gradient; but since there is at present no definite evidence either for or against such an assumption, attention is merely called to the possibility that it, as well as the physiological condition of the head-forming cells at different levels, may be concerned in determining head frequency.

The increase in effectiveness of the factor inhibiting head development with decrease in length of piece at a given body-level suggests either a decrement from the posterior cut surface anteriorly with a difference in steepness at different levels or an increase in intensity of the inhibiting factor with decrease in length of piece, also with a difference at different body-levels. Conceivably there may be a relation between intensity of the effect of section and size of isolated part of the nervous system but in the light of the evidence it seems probable that decrement in intensity or effectiveness from the posterior cut surface anteriorly rather than an intrinsic difference in intensity is concerned in the relation between head frequency and length of piece.

It is, of course, possible that effects of the posterior section inhibiting head development may pass anteriorly through other than nervous tissues, but it is believed that the evidence available indicates the nerve cords as the chief path of the inhibiting factor. In any case, it appears that the data on head frequency demonstrate the existence of a physiological gradient of some sort in *Euplanaria dorotocephala* and that they agree with other lines of evidence which indicate or show the existence of such a gradient.

That the head is a self-determining region is indicated; not only by the fact that its development may be inhibited by conditions arising in other parts of the piece, but also by the fact that when a head develops at any body-level it develops first from the cut surface and determines or induces the development of postcephalic parts anterior to the level from which the piece was taken (Child, 1911*b*, 1929). For example, a piece from the region just posterior to the mouth does not give rise first at its anterior end to a mouth and pharyngeal region, then to a prepharyngeal region, and finally to a head. A head develops directly from the anterior cut surface, and the prepharyngeal and pharyngeal regions are formed by the reorganization of parts of the piece posterior to the new head. Moreover, if the piece remains acephalic, this reorganization does not take place; no mouth, pharynx, or prepharyngeal region ever develops provided the piece was taken from the postoral level. In short, an isolated piece of the planarian body does not give rise to parts anterior to the body-level from which it was taken unless some degree of head development occurs; but even in complete absence of a head, it can give rise to all parts posterior to the level from which it was taken.

All the evidence at hand indicates that the condition essential for normal head development on an isolated piece is a degree of activation and dedifferentiation of the cells which give rise to the head sufficient to render them independent of, or insensitive to, correlative factors arising in other regions of the piece. If the correlative factors originating in other parts of the piece or from the posterior cut surface become, for any reason, sufficiently intense or effective, they may decrease or prevent the activation and dedifferentiation of the cells concerned in head formation. In that case

the region concerned does not become entirely independent and self-determining, and head development is more or less inhibited or entirely prevented.

In the intact planarian the cells of all postcephalic levels are capable of giving rise to a head (in most species) but are evidently prevented from doing so by their physiological relations with other regions of the body anterior and posterior to them. When the parts anterior to any level are removed by section, the physiological factors preventing head development which originate in those more anterior parts are eliminated. Apparently these are the most effective factors in preventing head development at postcephalic levels in the intact animal, since their removal usually results in head development unless the piece is short. But the cells at any given level of the body must also be subjected to physiological correlative factors originating at more posterior levels, and these factors also must play some part in maintaining the cells as a certain level of the intact body and preventing their activation and development into a head. The data on head frequency indicate that these posterior correlative factors are intensified temporarily by the posterior section but that their effectiveness decreases rapidly with increasing distance from the cut surface. In the longer pieces they do not prevent or decrease the activation of the head-forming cells, but in the shorter pieces they are effective.

In the various inhibited head-forms—teratophthalmic, teratomorphic, and anophthalmic,—the reduction or elimination of parts proceeds from the median region laterally. As regards this characteristic, it is suggested that for determination and development of the median region a higher degree of activation or dedifferentiation of the cells is necessary than for lateral regions, and that the differentially inhibited head-forms occur when none of the cells attain this degree. This suggestion is supported by the fact that the same differentially inhibited head-forms that are determined by physiological factors can be determined by external depressing agents (Child, 1912, 1916*a;* Behre, 1918; Buchanan, 1922). The fact that in many forms cells which are incapable under any known conditions of giving rise to a head are still able to give rise to a posterior end suggests that for head development in general a greater degree

of activation and dedifferentiation is necessary than for development of a posterior end.

The data presented in this paper do not support the view advanced by various authors that the head and other new parts develop from undifferentiated "formative" cells which remain quiescent and insensitive to correlative factors and are activated when section occurs. As already pointed out, the cells at the various postcephalic levels of the intact body are prevented from developing a head by their physiological relations with other parts. Since they are sensitive to conditions in their physiological environment, it is difficult to believe that they remain unspecialized, since the physiological environment must be different at different levels of the body. Moreover, in *Euplanaria dorotocephala* and in many other species neither their presence nor their number at a cut surface but relations between them and their physiological environment determine whether they give rise or do not give rise to a head, and also the character of the head when it does develop. When they do begin to develop a head, they change their appearance and behavior and give rise to tissue which appears to be embryonic and which undergoes differentiation. The only conclusion justified by the facts seems to be that cells which give rise to a head or to other parts in isolated pieces of planarians are to some degree specialized or differentiated cells until section occurs. When parts anterior to a particular body-level are removed, the physiological environment of these cells is altered and their behavior changes accordingly; they tend to dedifferentiate, become embryonic, and develop a head region as the first step in development of a new individual. But this dedifferentiation and development may be more or less completely inhibited by factors in their physiological environment which originate from more posterior levels; this occurs in the shorter pieces. In short, we do not believe that the hypothesis of undifferentiated "formative" cells as the basis of development of a head or any other part in isolated planarian pieces is in agreement with the experimental data.

The results of posterior half-section.—The very slight effect of posterior half-section on head development, even with repeated reopening of the section or with removal of tissue (Tables IV and V)

is of interest, since it indicates that the half-section does not prevent or inhibit the complete activation and dedifferentiation of at least some of the cells at the anterior cut surface, but merely retards head development slightly on the side of the half-section. The results of these experiments support the hypothesis advanced above that the inhibiting effect of the posterior section on head development is primarily nervous in character. The posterior half-section severs one nerve cord only. The experimental data indicate that on the side with intact nerve cord the cells at the anterior cut surface undergo complete activation and dedifferentiation, and therefore give rise to a head of normal pattern without the various degrees of differential inhibition characteristic of short pieces with complete posterior section. The fact that the median plane of the asymmetrical head usually does not coincide with the median plane of the piece but is shifted toward the intact side indicates that the cells of that side play the larger part in its formation. Since these heads develop as rapidly, except for the slight temporary retardation on the side of the half-section, and attain the same size as normal heads on long pieces, it appears probable that the cells of the intact side divide and grow more rapidly than in the usual case, in which they form only half of the head. This may be regarded as a reaction to the failure of the other side of the head to develop at the usual rate—that is, as a reaction to altered physiological environment.

If the inhibiting effect of posterior section were due to a chemical substance produced at the region of section and transported or diffusing anteriorly, it seems probable that it would determine the same differentially inhibited head-forms that appear after complete posterior section, but would be less effective because less in quantity than after complete section. Actually, however, the retarding effect is limited to the side of the half-section and differentially inhibited head-forms do not appear except in two cases among more than 500 pieces. Moreover, in the experiments with removal of a piece in connection with the half-section a substance inhibiting head development should be produced in much greater quantity and should therefore determine a higher frequency of differentially inhibited heads than in case of simple half-section; but, except for the two

cases mentioned above, only a higher frequency of temporary asymmetry appears in these experiments.

In short, the experimental data seem to us to indicate that the physiological factor which determines differential inhibition of head development in the shorter pieces of *Euplanaria dorotocephala* is primarily nervous in character and that it originates from the posterior section of the nerve cords and the activities following it in the posterior region of the piece. This factor apparently undergoes a decrement in effectiveness with increasing distance from the level of origin. The evidence indicates further that this factor acts by inhibiting to a greater or less degree the activation and dedifferentiation of cells in the anterior region of the piece which are directly concerned in head development.

Head frequency in other species.—In the natural environment the relation between head frequency, length of piece, and level of body differs in different species of triclads. In the species which undergo fission at a more or less definite post-oral level, the head-frequency curves are in general similar to those of *Euplanaria dorotocephala*. According to unpublished data on *E. novangliae* (Child) the decrease in head frequency from anterior to posterior levels of the anterior zooid is somewhat less for pieces representing a given fraction of body length, and in *E. lata* (Šivickis, 1923) still less than in *E. dorotocephala*. According to unpublished data on a Japanese species resembling *E. dorotocephala* and with a similar posterior zooid region, the head frequency in the natural water available at Sendai, Japan, was found to be much higher than in *E. dorotocephala*. Pieces one-fourth, one-sixth, and one-eighth of 18–20 mm. animals gave practically 100 per cent normal heads. Only in still shorter pieces did the inhibited head-forms begin to appear in pieces from the posterior region of the anterior zooid. According to data obtained by Dr. Minoura at Kyoto, animals which appear to be the same species as the Sendai form show a head frequency very similar to that of *E. dorotocephala*. Since head frequency may be greatly altered by changes in environmental conditions, it is by no means impossible that the same species may show very considerable differences in head frequency in the waters of different regions.

In the species which show no posterior zooid region and do not

undergo fission, the relation between head frequency, length of piece, and body-level differs from that of the species with posterior zooids. Abeloos (1930) has given data for the European *Planaria gonocephala* which indicate that head frequency decreases and death frequency increases in general from anterior to posterior levels in short pieces of the same length. In spite of the fact that his one-eighth pieces (Abeloos, Table XIII) show a much greater decrease in head frequency than one-third pieces (Abeloos, Table VI), he maintains that the decrease has no relation to length of piece. If this is true, *P. gonocephala* differs from all other species for which data are available. Šivickis (1933) has shown that in *P. lugubris* and in *Polycelis nigra* the time from section to appearance of eye-spots increases from anterior to posterior levels. In *Fonticola* (*Phagocata*) *gracilis* (Buchanan, 1933) head frequency decreases with decrease in length of piece and from anterior to posterior levels.

In the Dentrocoelidae for which data are available, development of a head occurs in normal environment only anterior to a level near the anterior end of the pharynx (F. R. Lillie, 1901; Šivickis, 1931). Unpublished data on a Japanese species of this family (Child) show that head development occurs a little farther posteriorly in that species (that is, about to the level of the mouth) and that development of a head can be induced in some cases at levels still farther posterior, though not in pieces from extreme posterior regions, by removal of the anterior ends of pieces which have developed as acephalic forms. According to Šivickis (1931), there is in *Dendrocoelum* a decrease in rate of head development from anterior to posterior levels, as indicated by time of appearance of eyes in pieces on which a head develops. The data on these forms indicate that at the more posterior body-levels the activation and dedifferentiation of the cells is not sufficient to permit head development but is sufficient for development of posterior ends.

All the evidence on head frequency and rate of head development in the various triclad species demonstrates beyond question the existence of a longitudinal physiological gradient of some sort which manifests itself in a decrease in rate of head development from anterior to posterior levels, in some species also in a decrease in head frequency in the same direction in pieces below a certain length, and

in the Dendrocoelidae in absence of head development at the more posterior levels. In the species which undergo fission at a definite post-oral level the posterior zooid region which represents one or more zooids and a posterior growing region show increase in head frequency from anterior to posterior levels. The only conclusion possible in the light of the facts seems to be that in these animals a longitudinal physiological gradient, which may or may not be present in all organs, is a factor in determining, directly or indirectly, rate and character of reconstitution at different levels in uniform normal environment.

It must be pointed out, however, that the relation between a pre-existing gradient and reconstitutional development is by no means invariable. The degree of activation and dedifferentiation of the cells at a cut surface apparently determines whether a head shall develop or not. If the pre-existing gradient differences in the piece are not great or if they are altered experimentally, a head may develop at the posterior end, a tail at the anterior end, or heads or tails at both ends. In fact, a head may develop in any region in which activation and dedifferentiation of the cells concerned is not prevented by their physiological or external environment. Among the annelids the relation between the growth gradient of the posterior region of the body and development of a head or posterior end differs in different species. In some a head, in others a tail, develops at the "low" anterior end of a piece from this region. In the one case, the cells concerned gain the upper hand, so to speak, become independent and dominant, give rise to a head and alter the pre-existing gradient; in the other, they are subordinated to the pre-existing gradient and develop in accordance with it. It seems probable that these differences depend primarily on conditions in the nervous systems of the species concerned.

In general the evidence at hand indicates that in reconstitution from isolated pieces in coelenterates, Turbellaria, annelids, and other forms the cells concerned must attain a certain physiological level in order to give rise to an apical region or a head. If they attain this level, they become dominant and independent and determine the gradient in other parts. If they are intrinsically incapable of attaining this level or if they are prevented from attaining it by their

physiological or external environment, their development may be determined by factors pre-existing in the piece and they may give rise to basal or posterior parts; or if they are incapable of reacting to the altered physiological environment by activation and dedifferentiation, they develop nothing.

SUMMARY

1. In *Euplanaria dorotocephala* head frequency at a given body-level decreases with decrease in length of piece.

2. In pieces below a certain length, head frequency decreases from anterior to posterior levels of the anterior zooid and increases again in the posterior zooid region.

3. Experiments on delay of anterior or posterior section show that in pieces of a given length from a given body-level head frequency increases with increase in delay of either section.

4. Removal of the anterior end in acephalic forms after the posterior end is fully developed is followed by development of normal heads; but after removal of the anterior end and transverse bisection of acephalic forms, the anterior pieces which have posterior cut surfaces again become acephalic and the posterior pieces without posterior cut surfaces develop normal heads.

5. A transverse posterior half-section at a very short distance from the anterior end of the piece does not prevent or differentially inhibit head development, but merely retards it slightly on the side of the half-section.

6. The experimental data indicate that the physiological factor inhibiting head development in the shorter pieces originates at the posterior cut surface in consequence of the changes following posterior section.

7. On the basis of the evidence the hypothesis is advanced that the inhibiting factor is primarily nervous in character and the result of section of the longitudinal nerve cords, though effects of section may perhaps also be transmitted from cell to cell in other tissues. The experimental data indicate that the inhibiting factor undergoes a decrement in effectiveness with increasing distance from the posterior cut surface and that the range of effectiveness varies in a definite way with body-level. They also indicate that this

factor inhibits head development by preventing complete activation and dedifferentiation of the cells which give rise to the head.

8. It is concluded that the data on head frequency do not support the hypothesis that the head or any other part develops from undifferentiated "formative" cells.

9. In other species of *Euplanaria* which undergo fission the relation of head frequency to length of piece and body-level is similar to that in *E. dorotocephala*, but the differences in frequency with level and length of piece differ in different species. In triclad species which do not undergo fission, rate or rate and frequency of head development decrease from anterior to posterior levels.

10. In the triclad species for which data are available rate of head development and head frequency in normal environment demonstrate the existence of a longitudinal physiological gradient of some sort, but the relation of head development to this gradient can be altered experimentally.

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RATE OF HEAD DEVELOPMENT AS INDICATED BY TIME OF APPEARANCE OF EYES IN THE RECONSTITUTION OF EUPLANARIA DOROTOCEPHALA.[1]

(Two figures)

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THE reconstitution of planarian pieces has been extensively studied in various species and by many authors. Head frequency (Child and Watanabe, 1935 and references there cited), scale of organization, and regenerative growth of new tissue have been investigated with special reference to the gradient theory, particularly in *Euplanaria dorotocephala;* and the data obtained have thrown light on the problems of physiological constitution and pattern in this organism. Head frequency is concerned with differences in capacity for, and degree of, head development; scale of organization, with the spatial scale and pattern of organization; regenerative growth, with rate of production and amount of new tissue. Besides these there is another important factor in reconstitution: the rate of differentiation of new tissue which may perhaps be regarded as representing an intensity factor in certain developmental processes. Data on the axial differential in rate of differentiation of the head as indicated by times of appearance of eyes have recently been given for four European species, *Planaria gonocephala* (Abeloos, 1927, 1930), *Dendrocoelum lacteum* (Šivickis, 1931), *Planaria lugubris*, and *Polycelis nigra* (Šivickis, 1933) and one American species, *Fonticola* (*Phagocata*) *gracilis* (Buchanan, 1933). In all of these species it has been found that time from section to appearance of eyes increases from anterior to posterior levels. The present paper is primarily concerned with rate of head differentiation in the earlier stages in relation to body-level and length of piece in *E. dorotocephala*, a species which differs from the other species studied in possessing a physiologically distinguishable posterior zooid region and in undergoing fission at the anterior boundary of this region.

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The paper by Child and Watanabe (1935) may serve to some extent as a general background for this paper.[2]

MATERIAL AND METHOD

The worms used as material were kept for at least 10 days under laboratory conditions, without food 5–7 days before experiment. In most cases lots of 25 pieces each were used and the experiment was repeated to a total of 100 pieces. For each such experimental lot worms 16–18 mm. in body length without recent fission were selected from a single stock. Pieces were cut with scalpel; and those from the same body-level were placed together in fresh well water in a finger bowl, covered, and kept in darkness without change of water. Temperature was controlled either by thermostat control of room temperature or, in summer, by surrounding the containers with running water. Temperature variation was not more than 2.0° C. in each experimental lot. When the eyes first appear near the boundary between new and old tissues, they lie somewhat ventral to the dorsal epithelium; consequently examination with compound microscope and strong transmitted light and careful focusing below the epithelium are necessary to determine their presence or absence in the early stages. Beginning 36 hours after section, pieces were examined every 12 hours and the number with eyes recorded.

From these numbers the arithmetical means of time were calculated. The mean times are, of course, only approximate, since pieces were examined at 12-hour intervals. For the posterior zooid region they are undoubtedly too large, because in pieces from that region the new eyes are distinguished with difficulty in early stages. Otherwise the means for different body-levels are comparable and serve as indices of the rate of regenerative development in its earlier stages. The statistical probability (P) that random sampling might give as great a mean difference in either directions as that observed was also calculated in those cases in which the data were sufficient, using the

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following formula and Student's table of t (Student, 1925, pp. 26–30):

$$t=\frac{\overline{D}}{\sqrt{\frac{\Sigma D^2-n\overline{D}^2}{n(n-1)}}},$$

in which n is the number of experimental lots in the series; D the differences of the means between two lots of pieces compared; and $\overline{D}$, the mean of D. Numerical values for developmental rates for a given body-level or length of piece are obtained by dividing the mean time taken as a standard by the mean time for the level or length of piece to be compared with it and multiplying the quotient by 100. In the tables the standard mean time is given the value 100, and rates given for other pieces are rates relative to this standard and appear as percentages of it.

Since pieces of equal length from different body-levels and pieces of different length with anterior ends at the same level were compared both length and level of piece are indicated numerically, the length by the fraction of the postcephalic length which the piece represents, 1/3, 1/4, 1/8, 2/8, 3/8, 4/8, and the body-level by a numeral or numerals following the fraction, 1/3–1, 1/3–2, 1/4–1, 1/4–2, 1/8–1, 1/8–2 etc., the body level 1 being the most anterior level immediately posterior to the head. Pieces of different length with anterior ends at the same level are designated as follows: 1/8–1, 2/8–1–2, 3/8–1–3, 4/8–1–4; 1/8–2, 2/8–2–3, 3/8–2–4, 4/8–2–5; etc. Pieces of equal length from different levels, but so long that they must be taken from different individuals, e.g., 3/8 pieces are 3/8–1–3, 3/8–2–4, 3/8–3–5, 3/8–4–6, and 3/8–5–7. The various pieces, lengths, and levels are indicated in Figure 1.

EXPERIMENTAL DATA

Series I. Times of appearance of eyes (*Table I*).—One hundred pieces of each third of the body were examined. All developed normal eyes. The mean times from section to appearance of eyes are given in Table I. Average mean times are 98.2 hours in 1/3–1, 106.4 hours in 1/3–2, and 104.4 hours in 1/3–3. Calculation of P gave 0.0014 for the difference of time between anterior and middle thirds and 0.019 for the difference between middle and posterior thirds.

These values indicate that the differences are highly significant. In Table I the rate in the middle third is 92 per cent; that of the posterior third, 94 per cent of that of the anterior third, taken as 100.

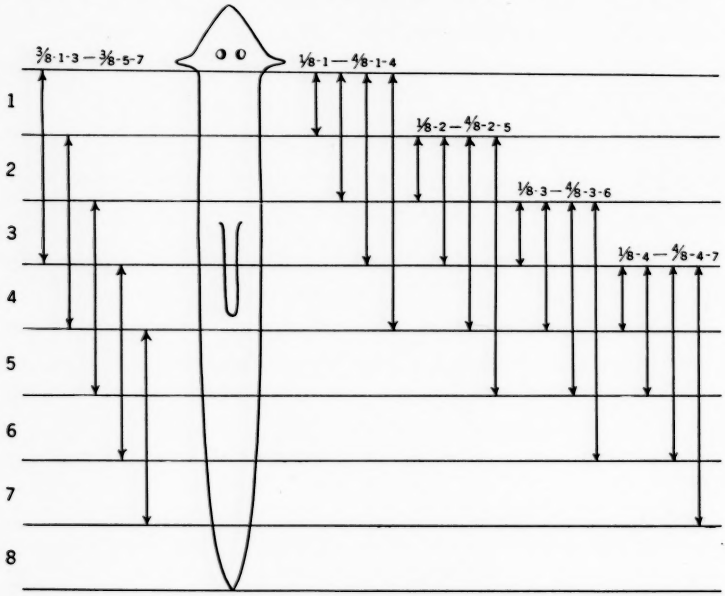

FIG. 1.—Diagram indicating pieces, lengths, and levels used

TABLE I

MEAN TIMES IN HOURS TO APPEARANCE OF EYES IN 1/3 PIECES (1/3–1 TO 1/3–3)

Each lot consists of 25 pieces for each level.

Lot	Temperature in Degrees C.	1/3–1	1/3–2	1/3–3
1	22.5–24.5	98.9	107.5	105.6
2	23.0–24.5	96.9	106.0	104.6
3	22.0–24.0	97.4	106.5	103.2
4	22.0–23.5	99.5	105.6	104.1
Av.		98.2	106.4	104.4
P		0.0014		0.019
Rates		100	92	94

Series II. Times of appearance of eyes in 1/4 pieces (Table II).—Anterior and posterior 1/4 pieces, 1/4–1 and 1/4–3 developed all normal eyes, while 1/4–2 and 1/4–3 developed both normal and abnormal eyes. But the percentage of normal as compared with abnormal eyes is high in these pieces. Of 100 pieces 1/4–2, 80 developed normal and 20 abnormal eyes; and of 100 pieces 1/4–3, 95 developed normal and 5 abnormal eyes. Mean times for 1/4 pieces for rates and values of *P* are given in Table II. The mean time values are lowest anteriorly (1/4–1), increase in the middle region (1/4–2, 1/4–3), and decrease again posteriorly (1/4–4); and the rates of development

TABLE II

MEAN TIMES IN HOURS TO APPEARANCE OF EYES IN 1/4 PIECES (1/4–1 TO 1/4–4)

Each lot consists of 25 pieces.

Lot	Temperature in Degrees C.	1/4–1	1/4–2	1/4–3	1/4–4
1	21.5–22.0	94.4	100.9	103.8	102.4
2	21.0–22.5	94.2	105.7	106.8	100.5
3	21.0–22.0	91.8	101.2	104.4	100.9
4	21.0–22.0	93.6	100.8	104.0	103.2
Av.		93.5	102.3	104.7	101.7
P		0.0023	0.0005		0.097
Rates		100	91	89	92

relative to the mean time of 1/4–1 as 100 decrease from anterior to middle region and increase again posteriorly. In other words, these values indicate clearly a U-shaped gradient. The values of *P* are respectively 0.0023, 0.0005, and 0.097, indicating that the differences between 1/4–1 and 1/4–2 and between 1/4–2 and 1/4–3 are highly significant, and those between 1/4–3 and 1/4–4 not significant. In each of the four series of Table II, however, the mean time for 1/4–4 is less than that for 1/4–3, though the difference is slight in two series.

Series III. Times of appearance of eyes in 1/8 pieces (Table III).—Since the frequency of abnormal eyes increases with decrease in length of piece, it seemed advisable to determine times of eye development in 1/8 pieces in which abnormal eyes are frequent for the

purpose of comparison with the data on longer pieces. The abnormal eyes in Table III include the types of eyes found in teratophthalmic and teratomorphic heads (Child, 1921), that is, eyes which are nearer the median line than normal and connected by a band of black pigment between the pigment cups, and superposed or single eyes in the median line. The pieces which develop no eyes have anophthalmic heads or are acephalic.

TABLE III

NUMBERS OF 1/8 PIECES (1/8–1 TO 1/8–8) WHICH DEVELOPED NORMAL EYES, ABNORMAL EYES, AND NO EYES; AND MEAN TIMES IN HOURS TO APPEARANCE OF EYES

Data of two series of experiments, each consisting of 25 pieces of each 1/8, are combined in the table, giving total of 50 pieces for each level. Temperatures, 19°–21° C.

Length and Level of Pieces		1/8–1	1/8–2	1/8–3	1/8–4	1/8–5	1/8–6	1/8–7	1/8–8
Normal eyes	Number of pieces	45	26	4	5	19	28	37	50
	Mean times	91.4	94.2	90.0	106.5	94.1	93.0	96.0	98.0
Abnormal eyes	Number of pieces	5	22	20	13	13	16	11	0
	Mean times	108.0	103.6	109.8	122.7	100.6	112.5	103.6	.....
No eyes	Number of pieces	0	2	26	32	18	6	2	0
All eyes	Number of pieces	50	48	24	18	32	44	48	50
	Mean times	93.0	98.7	106.5	118.0	99.8	100.0	97.6	98.0
	Rates	100	94	87	79	93	93	95	95

As regards frequency of normal and abnormal eyes, Table III shows that normal eye frequency (first section) and total eye frequency (fourth section) decrease from 1/8–1 to 1/8–3 or 1/8–4 and increase again from that level to 1/8–8 and that abnormal eye frequency and frequency of no eyes taken together increase in general from 1/8–1 to 1/8–3 and 1/8–4 and decrease again at more posterior levels. These differences show a U-shaped gradient similar to the head-frequency gradient (Child and Watanabe, 1935). The mean times for normal eyes and those for abnormal eyes show very considerable variation, but the mean times for all eyes in the fourth section of the table and the rates of development show very clearly

gradients corresponding to the head-frequency gradient. The table also shows, beyond question, that normal eyes develop more rapidly than abnormal at all levels except 1/8–8, where all eyes are normal. Further data concerning this point are given in the following series.

Series IV. Comparison of mean times and rates of development for normal, connected, and median eyes (Table IV).—This series gives a further analysis of times and rates for the different types of eyes which develop in the differentially inhibited heads of shorter pieces (Child, 1921). Normal eyes are the separate paired eyes characteristic of the species in nature; connected eyes are eyes approximated to the median line and connected by a band of black eye pigment and are found in the less extreme teratophthalmic heads; median eyes include those cases in which an apparently single eye develops in the median line instead of two eyes, and are found in the more extreme teratophthalmic and teratomorphic heads. For comparison of times and rates of development of these three types of eyes it is necessary, first, to use pieces from a single body-level, because rate of eye development is different at different levels, and, second, to choose pieces of a length and from a level which will give the three types in sufficient numbers to permit comparison. The second 1/8 pieces (1/8–2) are the most satisfactory for this purpose, since they give the three eye types in fairly comparable numbers. Of the one hundred 1/8–2 pieces of Table IV, 36 developed normal, 25 connected, 32 median eyes, and 7 no eyes. These experiments were performed during July and August; and, as Table IV shows, the temperatures were considerably higher (23°–27° C.), and the mean times are consequently considerably less than in Table III (19°–21° C.).

In each of the four lots of 1/8–2 pieces in Table IV the mean times are least for normal eyes, greater for connected eyes, and greatest for median eyes; and the differences in every case are large. The values of P show that the differences are highly significant. The rates of eye development, with normal eyes as 100, are 81 for connected and 64 for median eyes. These data indicate very clearly that the type of eye which develops is closely associated with the degree of retardation in development. Data on other lots of pieces of different length and from different body-levels show in general similar differences in mean times and rates of development of the different types

of eyes, but when the number of cases of any one type of eye is very small, individual variations sometimes alter the differences in mean times. For example, in some pieces of a lot connected eyes may develop as rapidly as normal eyes, while in other pieces the difference may be great; and if the number of normal or of connected eyes is very small, no difference or a difference in either direction may appear.

TABLE IV

NUMBERS OF PIECES WHICH DEVELOPED NORMAL, CONNECTED, AND MEDIAN EYES IN SECOND 1/8 PIECES (1/8–2), AND MEAN TIMES IN HOURS TO APPEARANCE OF EYES

Each lot consists of 25 pieces.

Lot	Temperature in Degrees C.	1/8–2							1/8–1	
		Normal Eyes		Connected Eyes		Median Eyes		No Eyes	Normal Eyes	
		No. of Pieces	Mean Times	No. of Pieces	Mean Times	No. of Pieces	Mean Times	No. of Pieces	No. of Pieces	Mean Times
1	25.0–27.0	9	61.3	3	80.0	13	114.6	0	25	60.0
2	23.0–24.5	11	67.6	9	81.3	5	101.6	0	25	61.4
3	24.5–26.0	8	60.0	6	72.0	8	97.5	3	25	59.0
4	25.0–26.5	8	66.0	7	77.1	6	88.0	4	25	59.5
Total		36		25		32		7	100	
Av.			63.7		77.6		100.3			60.0
P			0.0003			<0.0001				
Rates			100		81		64			

For comparison with the 1/8–2 pieces data for one hundred 1/8–1 pieces are given in the last two columns of Table IV. All of these pieces developed normal eyes; and the mean times for each lot are slightly lower, and the average (60 hours) is less than the average, 63.7 hours for the normal eyes of the 1/8–2 pieces. In other words, normal eyes develop more rapidly at the more anterior level 1/8–1 than at the second level 1/8–2—that is, the gradient in rate of development shown in preceding series appears here.

Comparison of the 1/8–2 pieces of Table IV with corresponding pieces of Table III shows certain differences for which no definite

reason can be given. The percentage with eyes is almost the same: 93 in Table IV, 96 in Table III; but the percentage of normal eyes is 36 in Table IV and 52 in Table III. The two experiments were performed with different stocks of animals from different localities, one collected in winter, the other in midsummer. The animals of Table III were subjected to laboratory conditions and feeding for a much longer time before using than those of Table IV. In general, head frequency and, of course, frequency, of normal eyes at a given body-level increases with length of time in the laboratory, and the differences which appear in the 1/8–2 pieces are such as to suggest that this factor may be concerned. On the other hand, head frequency increases with temperature, and the pieces of Table IV developed at a higher temperature than those of Table III. And finally, since only the two body-levels 1/8–1 and 1/8–2 were used in the experiments of Table IV, while in those of Table III all levels were used, there may be a slight difference in the length of the 1/8–2 pieces of the two tables, and this would alter the frequency of normal eyes (Child and Watanabe, 1935). But whatever the factors concerned, the differences are of no significance for the points which the tables are intended to show.

Series V. Effect of length of piece on rate of development (Table V).— Table III shows that in pieces from body-levels which give high head frequency, rate of development is higher than at body-levels which give low head frequency. Moreover, head frequency at a given body-level decreases as length of piece decreases (Child and Watanabe, 1935). In view of these facts, it seemed desirable to determine rates of development in pieces of different length with anterior ends at the same level and to extend such comparison to different body-levels. Table V gives rates of development relative to 1/8–1 as standard for pieces of four lengths, 1/8, 2/8, 3/8, and 4/8, from body-levels 1, 2, 3, and 4 (Fig. 1). Rates of development for four lots of 25 pieces each for each length of piece and each body-level are given in the columns I–IV of the table and the average rates in the following column. The data for the pieces of different length with anterior ends, at the same body-level in a given lot, were obtained at the same time on pieces from animals of the same stock; but the data for different body-levels of the same lot and for the

same body-level of different lots were obtained at different times, in many cases from different stocks and development occurred at different temperatures. Consequently, only the four rates for pieces of different length and for a given body-level of a given lot are directly comparable with each other. The data of the columns I–IV show, first, that at the anterior level 1 the rates of development are almost

TABLE V

RATES OF DEVELOPMENT AT FOUR BODY-LEVELS, 1–4, IN RELATION TO LENGTH OF PIECE

Rates are determined from mean times for 100 pieces of each length at each level, with the mean time of 1/8–1 pieces as standard for each lot.

Body Levels Compared	Length of Pieces	Experimental Lots				Average Rates	Number of Pieces		
		I	II	III	IV		Normal Eyes	Abnormal Eyes	No Eyes
1	1/8	100	100	100	100	100	100	0	0
	2/8	97.7	101.7	100.8	99.1	99.8	100	0	0
	3/8	100.8	100.0	99.3	99.1	99.8	100	0	0
	4/8	96.9	99.1	100.0	99.1	98.7	100	0	0
2	1/8	65.5	76.6	77.1	78.1	74.3	36	57	7
	2/8	89.0	82.7	80.6	88.8	85.2	52	46	2
	3/8	91.7	95.9	96.1	94.7	94.6	100	0	0
	4/8	90.9	95.9	96.2	95.5	94.6	100	0	0
3	1/8	88.6	87.7	81.6	81.9	84.7	17	37	46
	2/8	90.8	85.6	82.0	87.6	86.0	50	44	6
	3/8	88.4	89.9	90.8	90.7	89.0	96	4	0
	4/8	90.3	88.8	90.8	90.4	90.0	100	0	0
4	1/8	81.2	83.3	79.2	83.0	81.6	50	36	14
	2/8	86.0	85.3	86.7	86.5	86.1	72	24	4
	3/8	88.4	87.6	86.0	88.9	87.7	99	1	0
	4/8	89.0	88.6	87.0	87.4	88.0	100	0	0

identical for pieces of different length, and, second, that at the levels 2, 3, and 4 there is in general a considerable increase with increase in length of piece from 1/8 to 3/8 and less or no increase in rate with increase in length from 3/8 to 4/8. In a few cases the increase is slight, or longer pieces may show a slightly lower rate than the next shorter pieces; but in each group of four values for pieces of different length at a given body-level except at level 3 in lot I and II, the increase in rate appears. The average rates for levels 2, 3, and 4 show

the increase in rate with increase in length from 1/8 to 3/8 in all cases.

In the last three columns of Table V the numbers of pieces of each length at each level which develop normal, abnormal, or no eyes are given. These data show that at levels 2, 3, and 4 the frequency of normal eyes increases with increase in length of piece.

It should be noted that the four different levels of eye development included in Table V represent approximately the anterior half of the planarian body; that is, they are all within the anterior zooid. They correspond to the descending portions of the head-frequency curves in Figures 1–4 of the paper by Child and Watanabe preceding this; and a comparison of Table V and the head-frequency data for different body-levels and lengths of piece shows that relation of head frequency and rate of head development to length of piece is the same. Differences in rate of development at different body-levels are less distinct than differences in head frequency, in part because many of the shorter pieces from the more posterior levels do not develop eyes at all; but even in the miscellaneous data of Table V the rate of development in the 3/8 and 4/8 pieces decreases from anterior to posterior levels in all lots.

Series VI. Times of appearance of eyes in 3/8 pieces from different body-levels (*Table VI*).—The preceding series have shown that rate of eye development differs with body-level and length of piece and that rate of development for abnormal eyes is lower than for normal. The 1/3 and 1/4 pieces of Series I and II develop almost entirely normal eyes, but in shorter pieces the frequency of abnormal eyes and eyeless forms increases at the more posterior levels of the anterior zooid. In the present series pieces 3/8 of the post cephalic length are used in order to eliminate with still greater certainty length of piece as a factor in the rate of development. The body-levels used are 1, 2, 3, 4, and 5 (Fig. 1), that is, the levels of the 3/8 pieces differ by 1/8 of the postcephalic length. The mean times are given in Table VI. All pieces of each lot were cut on the same day. The experiments were performed during July and August at relatively high temperatures, so that the mean times are short. The 3/8 pieces from different levels are distinguished in the table by numerals indicating the eighths of the body length which they include. The anterior

3/8 is 3/8–1–3; it includes the first to the third 1/8; the second 3/8 is 3/8–2–4, etc. The 3/8 pieces of any one lot were necessarily taken from different individuals; but mean times for eye development were also determined for 1/8–1 pieces of the same individuals

TABLE VI

MEAN TIMES IN HOURS TO APPEARANCE OF EYES IN 3/8 PIECES FROM FIVE BODY-LEVELS

Each lot consists of 125 pieces, 25 for each level. Mean times for 1/8–1 pieces from same individuals added for comparison

Lot	Temperature in Degrees C.	3/8–1–3	3/8–2–4	3/8–3–5	3/8–4–6	3/8–5–7	1/8–1
1	25.0–27.0	60.4	62.4	64.8	66.7	67.2	58.7 59.5 59.0 59.5
2	24.5–26.5	59.0	62.8	67.2	67.2	69.1	60.4 59.5 58.5 60.4
3	24.0–26.0	59.5	62.8	65.8	68.1	70.5	59.5 59.5 59.5 59.5
4	26.0–28.0	57.1	58.4	64.3	65.3	65.2	56.6 57.6 57.6 56.6
Av.....	.....	59.0	61.6	65.0	66.8	68.0	58.6
P.....	.....	0.022	0.016	0.088	0.124		
Rates.....	.....	100	96	91	88	87	

from which the 3/8 pieces were taken, except in the case of the anterior 3/8, 3/8–1–3, where this was impossible. The mean times for the 1/8–1 pieces are given in the last column of Table VI, and times for 3/8 and 1/8 pieces which are in the same horizontal line of the table were obtained from pieces of the same animals.

Table VI shows an increase in mean time from anterior to pos-

terior levels in all except two cases—Lot 2, 3/8–3–5 and 3/8–4–6, and Lot 4, 3/8–4–6 and 3/8–5–7—and the average mean times show a continuous increase and the average rates a continuous decrease. The values of *P* indicate, however, that the differences for the first and second and the second and third 3/8 pieces are highly significant, while those for the third and fourth are less, and those for the fourth and fifth still less significant.

As the last column of Table VI shows, the mean times for the 1/8–1 pieces from the same individuals as the second to the fifth 3/8 pieces differ but little from each other and from the mean times for the 3/8–1 pieces, and are in all cases less than the mean times for the other 3/8 pieces. These points confirm further the evidence from Table V, that at the anterior body-level length of piece within the limits used has little effect on rate of development, and also supplements the evidence from the 3/8 pieces of Table VI in indicating that eyes develop more rapidly at the anterior level than at other levels of the anterior zooid, even though the anterior pieces are shorter than the others.

Graphic representation of developmental rates.—In Figure 2 the rates of development for Tables I and II, for all eyes in Table III, and for Table VI are plotted as ordinates against body-levels as abscissae. These are rates for 1/3 pieces (unbroken line), 1/4 pieces (dashes), and 1/8 pieces (dots and dashes) from all body-levels including the posterior zooid region and for 3/8 pieces (dotted) from the anterior level to the region of the fission zone. The curves show that the decrease in rate in the anterior zooid is greatest in the 1/8 pieces. The increase in rate in the posterior zooid region is less regular, probably because of the difficulty in distinguishing the early stages of the eyes. The changes in slope of the 1/8 curve in the posterior region suggest the presence of a third zooid at about 1/8–6. The 3/8 curve for the anterior zooid, representing a different stock from a different locality and development at a different temperature, shows in general lower rates than the 1/3 and in its posterior part lower than the 1/4, but resembles the other curves in the regular decrease in rate in the anterior zooid.

It should be noted that the standard rate taken as 100 for all the curves represents a slightly different mean time in the 1/3, 1/4, and

1/8 series and a much shorter time in the 3/8 series. In other words, the curves represent relative rates as percentages of the standard.

Comparison of this graph with the graphs of head frequencies in the paper preceding this (Child and Watanabe, 1935) shows a fairly

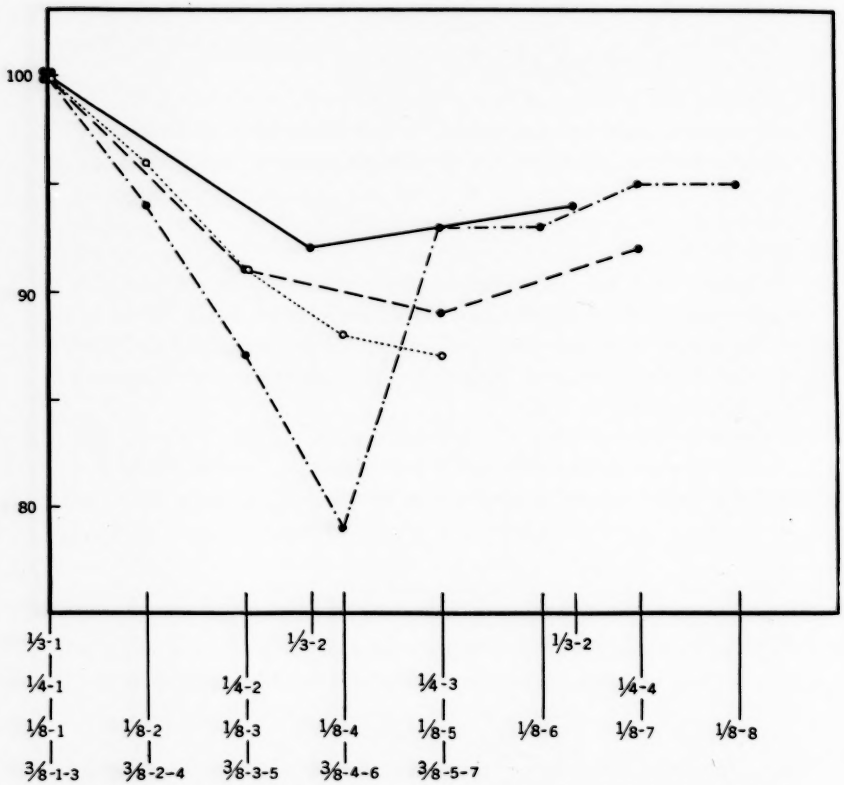

FIG. 2.—Rates of development plotted as ordinates against body-levels as abscissae. One-third pieces (unbroken line), 1/4 pieces (dashes), and 1/8 pieces (dots and dashes) from all body-levels; and 3/8 pieces (dotted line) from anterior level to fission zone.

close correspondence as regards course of the curves in relation to body-level and in the 1/3, 1/4, and 1/8 pieces with respect to length of piece.

In view of the difficulty in distinguishing the earliest stages of eye

pigment even in the new tissue, and of the fact that the mean times, and therefore the rates, are only approximate, the similarity of the curves of rate and head frequency suggests that the same physiological differential underlies both.

TABLE VII

STANDARD DEVIATIONS IN HOURS OF MEAN TIMES OF TABLES, I, II, AND VI

PIECES	EXPERIMENTAL LOTS				AVERAGE STANDARD DEVIATIONS WITHIN LOTS
	1	2	3	4	
1/3–1	11.6	10.3	8.4	10.3	10.3
1/3–2	11.2	10.7	11.1	12.0	11.2
1/3–3	7.7	10.1	7.7	9.6	8.8
1/4–1	8.0	6.9	7.0	7.3	7.3
1/4–2	9.8	9.1	7.3	9.2	8.9
1/4–3	10.0	8.4	8.7	9.6	9.1
1.4–4	7.6	6.1	7.5	6.0	6.8
3/8–1–3	7.3	3.3	6.4	5.5	5.8
3/8–2–4	5.3	5.0	5.2	5.4	5.2
3/8–3–5	6.8	6.0	7.9	7.6	7.1
3/8–4–6	6.9	6.2	8.4	8.6	7.6
3/8–5–7	6.9	11.6	7.2	9.3	9.0

Standard deviations in this table are calculated according to the formula:

$$\sqrt{\frac{\Sigma d^2}{n-1}};$$

and average standard deviations, according to the formula:

$$\sqrt{\frac{1}{4}\left(\frac{\Sigma d_1^2}{n_1-1}+\frac{\Sigma d_2^2}{n_2-1}+\frac{\Sigma d_3^2}{n_3-1}+\frac{\Sigma d_4^2}{n_4-1}\right)}.$$

In both formulas, n is the number of pieces and d the deviation either positive or negative of times of observation from the mean times of each lot. In the second formula n_1-n_4 and d_1-d_4 are, respectively, numbers of pieces and deviations for each lot.

Variation in mean times to appearance of eyes at different body-levels.—In Table VII standard deviations of the mean times and average standard deviations are given for 1/3, 1/4, and 3/8 pieces. The table shows that, with some exceptions, variation in mean time, (that is, in rate of development to appearance of eyes) increases from anterior to posterior levels of the anterior zooid and decreases again in the posterior zooid region. The pieces 1/3–3 and 1/4–4 are wholly in the posterior zooid region and show in all cases a lower

standard deviation than the pieces 1/3–2 and 1/4–3 next anterior to them. The posterior portions of pieces 1/4–3, 3/8–4–6, and 3/8–5–7 are in the posterior zooid region, but the level at which the head develops is in the anterior zooid. The table does not indicate a definite effect of the presence in these pieces of a part of the posterior zooid region on the variation in rate of development. The question of possible factors concerned in the variation at different body-levels is considered below.

DISCUSSION

The experimental data as evidence for a physiological gradient.— The mean times from section to appearance of eyes and the numerical values for rates of development expressed as percentages of a mean time taken as representing a standard rate differ in a definite and orderly manner with level of body and at certain body-levels with length of piece. It should perhaps be pointed out that the values for rate of development involve no assumptions as to constancy of rate from section to appearance of eyes. They merely show certain relations of the mean times and signify only that something in the series of changes between section and appearance occurs more rapidly in some pieces than in others.

The data of Tables I, II, III, and VI show that in the regenerative development of the head of *Euplanaria dorotocephala* the time from section to the appearance of normal paired eyes increases from anterior to posterior levels of the anterior zooid. They also indicate that this time decreases again in the posterior zooid region but the decrease is not always statistically significant. Undoubtedly, however, the observed times for levels in the posterior zooid region are too great in comparison with those from more anterior levels, because in the posterior zooid region the earlier stages of eye development occur beneath the pigmented layer of the old tissue and are very difficult to distinguish. Since normal eyes occur only in heads which are normal, not differentially inhibited, these differences in rate may be regarded as differences in rate of early development of normal heads. Table III shows that abnormal eyes appear later than normal eyes, and Table IV that median eyes appear later than connected paired eyes. These abnormal eyes are

characteristic of the differentially inhibited teratophthalmic and teratomorphic head forms. The data indicate that these differentially inhibited heads develop more slowly than normal heads and that the heads with a median eye which represent a greater degree of differential inhibition develop more slowly than the less inhibited heads with connected eyes, and these more slowly than normal heads.

At the anterior body-level immediately posterior to the head, length of piece between 4/8 and 1/8 has little or no effect on rate of development; but at more posterior levels of the anterior zooid, rate of development decreases as length of piece decreases from 3/8 to 1/8 (Table V). These differences in rate of development are in large part due to the fact that the frequency of heads with abnormal eyes increases as length of piece decreases and as the level becomes more posterior in the anterior zooid. In short, the decrease in rate of development parallels, in general, the decrease in head frequency from anterior to posterior levels of the first zooid (Child and Watanabe, 1935). Data for the posterior zooid region indicate that developmental rate increases again there, as does head frequency; but the significance of these data is uncertain.

It appears, then, that the data of the present paper on rate of development indicate the existence of a physiological gradient, at least in the anterior zooid, corresponding to the head-frequency gradient; that is, rate of development even of heads with normal eyes decreases in the same direction and over the same region of the body as head frequency and shows a relation similar to that of head frequency to length of piece. The experimental data of the preceding paper by Child and Watanabe indicate that the physiological factor determining head frequency in relation to body-level and length of piece is the degree of activation and dedifferentiation of the cells concerned in head development. According to this view, if activation and dedifferentiation are inhibited to a greater or less degree or prevented by physiological effects of posterior section or by external factors, head development is inhibited or prevented. The present paper shows not only that development is less rapid in inhibited than in normal heads but also that development of normal

heads occurs least rapidly in those regions of the body in which head development is most inhibited by physiological factors.

In the light of the evidence it is suggested that the chief factors in determining the differences in rate of development are rate and degree of activation and dedifferentiation of the head-forming cells at different body-levels and in different lengths of pieces. If activation and dedifferentiation are complete, normal heads develop; and it may be that further development of normal heads occurs at the same rate at all levels. If activation and dedifferentiation are retarded, the rate of development, as indicated in the present paper, is decreased; and it may be that the rate of further development of the inhibited head which results is also lower than that of the normal head.

The general parallelism in the relation of rate of development and head frequency to length of piece and body-level in the shorter piece, and the fact that differentially inhibited heads develop less rapidly than normal indicates that the same factor is concerned in both. The evidence given in the paper preceding this indicated that this factor originates from the posterior section, that it is probably nervous in character, that it is effective in inhibiting development when the piece is below a certain length, and that this length differs in a definite way at different body-levels.

Rates of head development in other species.—No data are at hand for other triclad species which have a posterior zooid region and a definite fission zone; but in several which do not undergo fission and show no evidence of a posterior zooid region the rate of head development, as indicated, has been found to decrease from anterior to posterior levels over the whole body length. According to Abeloos (1927, 1930), this is the case in *Planaria gonocephala*, which he says behaves as a single zooid. Abeloos has determined times from section to different stages of eye development (eyes scarcely indicated; eyes distinctly indicated; eyes clearly marked, reniform) but has used only small numbers of pieces; and his criteria of the different stages of eye development are rather difficult to apply practically, at least in *Euplanaria dorotocephala*.

Abeloos (1930) maintains that rate of eye development and frequency of abnormal eyes have no relation to length of piece; but

comparison of his Tables VI (1/3 pieces) and XIII (1/8 pieces) shows a higher frequency of abnormal eyes and acephalic forms in the latter, although he states that these are as frequent in longer pieces. His data do not seem to support his conclusion, and in any case appear insufficient to exclude the possibility of a relation between length of piece, rate of development, and frequency of abnormalities.

Šivickis (1931) has found that in *Dendrocoelum lacteum* time from section to appearance of eyes increases from anterior to posterior levels of the prepharyngeal region, heads being absent at more posterior levels. In a later paper Šivickis (1933) has shown that in *Planaria lugubris* mean times from section to appearance of eyes increase from anterior to posterior levels throughout the body length and that in *Polycelis nigra* mean times for anterior and middle level show a similar difference. Length of piece in relation to head development is not considered. Unfortunately, in many cases Šivickis' observations of pieces for recording numbers with eyes were made at very different and unequal time-intervals in different series of experiments; consequently the mean times to appearance of eyes are comparable only for the pieces of a single series and most of his conclusions drawn from comparison of different series are either invalid or doubtful. The mean times to appearance of eyes at different body-levels are not open to this criticism, since they are determined from single series. All those of his tables which give data for pieces of equal length from the different body-levels show the anteroposterior gradient in time to appearance of eyes, but the mean times of different tables are in most cases not comparable.

Buchanan (1933) finds a similar anteroposterior developmental gradient in *Fonticola* (*Phagocata*) *gracilis*. He concludes that rate of head development has no relation to length of piece, though his Table I clearly indicates a relation similar to that in *Euplanaria dorotocephala*, except that the decrease in rate extends over the whole body length. At the anterior level, the time of appearance of eyes is the same in 1/2, 1/4, and 1/8 pieces, but the increase in time at the more posterior levels increases with the decrease in length of piece.

It is to be expected that the effect of length of piece on rate of development will differ in different species as the effect on head frequency does (Child and Watanabe, 1935). In some species the rate at a given level will be decreased in longer pieces than in others; but it is probable that in most, if not all, species of this group which are capable of developing a head at all levels, a relation between length of piece and rate of development will be found at the more posterior levels.

Variation in rate of development.—The mean times from section to appearance of eyes differ in different lots of pieces and series of experiments. Some of these differences are undoubtedly due to temperature, the mean times being less at higher than at lower temperatures. Differences in physiological condition of different stocks collected at different times of year and kept for different periods in the laboratory may perhaps also be concerned. Table VII indicates, however, that the variation increases in general from anterior to posterior levels of the anterior zooid. While the conditions determining this variation are not known with certainty, its relation to body-level suggests that the physiological gradient is in some way concerned. Certain possible factors may be briefly mentioned. The presence of the posterior zooid region in *Euplanaria dorotocephala* indicates that the dominance of the head region is not completely effective in maintaining that region as a part of the individual. Probably the posterior region of the anterior zooid is less completely subordinated than more anterior regions. As a matter of fact, evidences of some degree of physiological isolation anterior to the fission zone and extending anteriorly into the pharyngeal region have been found in some long animals, but different individuals even of the same length differ in this respect. But even if actual physiological isolation does not occur in this region, it is less immediately under control of the dominant region and apparently more influenced by regions posterior to it than are more anterior levels. It is suggested then that the greater variation in mean times in this region is associated with the greater variation in physiological conditions to which it is subjected because of its position in the body.

According to Abeloos (1930, p. 69) the individual variations in

times of differentiation of eyes increase in *Planaria gonocephala* from anterior to posterior levels through the whole length of the body; and the data given by Šivickis (1933) for *P. lugubris* indicate similar conditions in that species also, although some irregularities appear.

The posterior zooid region of Euplanaria dorotocephala.—In the longer individuals this region is complex and may consist of two, three, perhaps in some cases even more, zooids and a posterior growing region (Child, 1910, 1911b, 1913, 1930). Posteriorly from the chief fission zone each successive zooid apparently shows a short anteroposterior gradient, but at higher physiological levels than the zooid anterior to it. Consequently the gradient in the posterior zooid region is in general ascending posteriorly; but when it is analyzed by isolation of short pieces (Child, 1911*b*) and by differential susceptibility (Child, 1913, 1930), the different gradients appear. In other words, the right arm of the U-shaped gradient representing the posterior zooid region of *E. dorotocephala* consists of several short anteroposterior gradients which differ in position and probably in number in different individuals of the same length and the difference in physiological condition in each gradient is so slight that the separate gradients do not become evident in the more rapidly lethal agents or in the longer pieces from this region. Because of the complexity of this region, the frequency of fission in pieces isolated from it, and the difficulty of distinguishing the early stages of eye development, determination of times and rates of development is less complete for this region than for the anterior zooid. So far as they have been examined, other triclad species—*Planaria lata* (Šivickis, 1923), *E. novangliae*, *E. maculata*, and *P.* sp. Japan (Child, 1932)—which undergo fission at a definite postoral zone show a gradient similar to that of *E. dorotocephala;* but in the species which do not undergo fission—*P. gonocephala* (Abeloos, 1927,1930), *P. lugubris* (Šivickis, 1933), *Fonticola gracilis* (Buchanan, 1927, 1933), and *Curtisia foremanii* (Child, unpublished)—there is no evidence of a posterior zooid region. Head frequency, scale of organization and rate of development decrease posteriorly over the whole body length.

Conclusion.—The data on differential susceptibility to lethal

agents and differential tolerance to lower concentrations (Child, 1912, 1913, 1930), on respiration (Hyman, 1923; Robbins and Child, 1920), on rate of growth of new tissue in regeneration and scale of organization under natural and experimental conditions (Child, 1911a, 1912; Buchanan, 1927), and on head frequency (Child and Watanabe, 1935) all agree not only in indicating the existence of a longitudinal physiological gradient in *Euplanaria dorotocephala* but also as regards its general form. In other words, all these lines of evidence indicate that certain physiological conditions decrease from anterior to posterior levels and increase again, though probably not continuously, in the posterior zooid region. The data of the present paper are also in agreement with the other data as regards the anterior zooid, since they show that rate of head development decreases from anterior to posterior levels. Apparently it also increases again in the posterior zooid region, but the data do not show this with certainty.

These various physiological differentials all appear to be aspects or expressions of a physiological differential or gradient which is a fundamental characteristic of the longitudinal axis. There is no evidence that the axis is anything other than this gradient.

The evidences of a gradient which appear in the earlier stages of reconstitution of pieces from different body-levels in the planarians and in other forms are evidences of the gradient of the individual from which the pieces were taken. With the determination of a new dominant region—the head, in planarians—the gradient is altered and a new developmental pattern appears on a different scale. The localization of the dominant region and of the posterior end may be determined by the original gradient or in some cases by other factors which are more effective than the gradient, and the new developmental pattern may be in part determined by the old and in part by the new gradient, or in some cases the old gradient may be entirely obliterated. As the development of the new individual from the piece progresses, the altered or new gradient becomes more and more the determining factor, and rates of growth and differentiation in different regions may become very different from those of earlier stages. For example, the head region grows and develops more rapidly than the posterior end in earlier stages, but later the latter

may grow more rapidly than the head. But the evidence indicates that the physiological basis of the developmental pattern is the gradient. This makes possible the realization of different hereditary potentialities in different parts of the mass. Reconstitutional development is of particular interest for the study of physiological gradients, since it enables us to learn something about the part which the original gradient plays in determining the new pattern and the changes which it undergoes as the new pattern is determined.

SUMMARY

1. Mean times from section to appearance of eyes were determined in pieces of *Euplanaria dorotocephala* of equal length from different body-levels and in pieces of different length from the same level. From these mean times values for rates of development from time of section to appearance of eyes were obtained as percentages of a standard mean time taken as 100.

2. In pieces of equal length the rate of development of heads with normal eyes decreases from anterior to posterior levels of the anterior zooid and apparently increases again in the posterior zooid region. This gradient corresponds closely to the head-frequency gradient.

3. Heads with abnormal eyes (connected and median eyes) which appear in the shorter pieces from the more posterior levels of the anterior zooid develop less rapidly than those with normal eyes, and heads with a median eye less rapidly than heads with connected eyes.

4. In pieces of different length from the same body-level the relation between rate of development and length of piece differs at different body-levels. At the anterior level, rate is little or not at all altered by difference in length of piece, but at more posterior levels of the anterior zooid, rate increases as length of piece increases from 1/8 to 3/8 of the postcephalic body length. The relation between length of piece and rate of development corresponds closely to the relation between length of piece and head frequency. It is suggested that the same physiological factor that determines differentially inhibited head forms in the shorter pieces also retards the early development of the head.

5. The observed differences in developmental rate agree in gen-

eral with the evidence on differential susceptibility, respiration, scale of organization, and head frequency in indicating the existence of a physiological gradient in the longitudinal axis of *E. dorotocephala*.

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FERTILIZATION MEMBRANES OF CENTRIFUGED ASTERIAS EGGS

I. THE EFFECTS OF CENTRIFUGING BEFORE FERTILIZATION

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(One plate)

THERE has been considerable controversy concerning the origin of the fertilization membrane in marine invertebrate eggs. For the extensive literature on this subject see Gray (1922), Hyman (1923), and Hobson (1927, 1932). The majority of the workers in this field believe that the unfertilized eggs of many marine invertebrate animals are surrounded by a thin surface layer, discontinuous with the fluid protoplasm, which is known as the vitelline membrane. There is much evidence to show that in certain marine forms this layer becomes separated from the protoplasmic surface of the egg at the time of, or shortly after, fertilization, and then becomes the fertilization membrane. At the protoplasmic egg surface the hyaline plasma membrane subsequently appears. Between the fertilization membrane and the hyaline plasma layer lies the perivitelline space. It is also generally recognized that there is a great change in the physical and chemical properties of the fertilization membrane following the formation of the perivitelline space. Some of the physical changes are visibly apparent in the thickening and hardening of the membrane (see A. R. Moore, 1930, Hobson, 1927). The observations to be presented are primarily concerned with the mechanism by which the fertilization membrane becomes separated from the egg surface.

MATERIAL AND METHODS

As material, the eggs of the starfish, *Asterias forbesii*, were used. Under ordinary conditions the full-grown egg of this animal remains immature, with its germinal vesicle intact, until it is removed from the body cavity of the female to sea water. About 10 minutes later

the nuclear membrane begins to disappear, and in about 60 minutes the first polar body is separated. The second polar body appears about 20 minutes after the first. Concurrently with the nuclear maturation there exists a cytoplasmic maturation which appears to result from the diffusion of the nuclear sap into the cytoplasm following the destruction of the membrane of the germinal vesicle. This cytoplasmic maturation renders the egg fertilizable (Delage, 1901). Subsequent to the breakdown of the germinal vesicle, and before the formation of the first polar body, there occurs a critical stage when the egg shows itself to be particularly sensitive to external influences.

By means of the centrifuge it is possible to study certain physical properties of the egg, and to compare the changes in these physical properties with the changes in the fertilizable condition of the egg during the maturation cycle. Lyon (1907) has indicated that there is a decrease in protoplasmic viscosity accompanying the breakdown of the germinal vesicle. In addition to viscosity changes the variation in the rate of pulling apart of the egg into fragments under centrifugal force may be studied. Generally speaking, the periods of maximum rate of fragmentation under centrifugal force correspond very clearly with the periods of maximum fertilizability. These periods are probably also the times of minimum viscosity, though differences in this latter property are not at all striking. The details of these changes will be the subject of a later paper. For the present, the discussion will be confined to the effects of centrifuging unfertilized eggs at these different periods of maturation upon subsequent fertilization and membrane separation.

The eggs were centrifuged in an electric centrifuge giving a force of approximately 6,000 times gravity, with a layer of isotonic sucrose at the bottom of the centrifuge tube. Due to the column of liquid centripetal to the eggs, the pressure developed at the egg layer while centrifuging is approximately 614 pounds per square inch. The temperature of the centrifuge tubes was about one degree above room temperature (19° to 26° C.) after any duration of time of centrifuging greater than 30 seconds.

THE CENTRIFUGED EGG

Before the breakdown of the germinal vesicle, the egg normally shows little tendency to stratify or elongate with 10 minutes of cen-

trifuging. The germinal vesicle migrates centripetally, while the nucleolus moves toward the centrifugal pole, forming a pocket-like depression by stretching the nuclear membrane (see Fig. 13). An occasional lot of eggs can, however, be stratified in this stage by prolonged centrifuging (30 to 40 minutes). These show an oil cap centripetal to the germinal vesicle, and a narrow clear zone at the sides. The yolk spheres occupy the remainder of the egg. Centrifuging decreases the percentage of eggs with intact germinal vesicles, as Lyon pointed out, yet the nuclear membrane may in particular instances be considerably elongated by the centrifugal-ward migrating nucleolus, without breakdown occurring.

Immediately after the breakdown of the germinal vesicle the egg is stratified by ten minutes of centrifuging into three layers: a narrow centripetal oil cap; a wider clear zone (not truly hyaline, and about equal in volume to the fluid previously contained in the germinal vesicle); and a wide zone of yolk spheres comprising more than 75 per cent of the egg volume. Shortly after this period the same amount of centrifuging results in the stratification, elongation, and separation of the egg into two fragments inside of the jelly-hull. The centripetal light fragment contains the oil, clear zone, and some yolk spheres. The centrifugal fragment contains the remainder of the yolk. The significance of the change in rate of pulling apart of the egg under centrifugal force cannot be discussed at present, but the swelling of the jelly, changes in the protoplasmic viscosity, in the number of granules, and in the egg cortex are probably all involved. Centrifuging for a longer time at the same stage may tear one or both fragments out of the jelly, but for the fertilization experiments it is advantageous to keep them together. It is usually the centripetal fragment which is pulled free of the jelly, while the centrifugal fragment tends to retain the jelly-hull. At the time that the first polar body is visible, the egg shows a little less tendency to pull apart under centrifugal force, and this is followed by a slight increase and then a decrease as the second polar body forms. In the mature egg Lyon (1907) previously observed but two zones; a small clear area at the lighter pole, and a wide, granular zone. The nucleus of the centrifuged mature egg lies in the clear zone.

THE FERTILIZATION OF CENTRIFUGED EGGS

After subjecting the eggs to centrifugal force, they were inseminated, and some of the phenomena accompanying fertilization followed in the living egg. Eggs which have been centrifuged but not inseminated are not activated to membrane separation.

Eggs which have been centrifuged in the germinal vesicle stage, and in which the germinal vesicle has remained intact (Fig. 13), sometimes, upon insemination, separate a fertilization membrane. This fertilization membrane is only slightly thinner at the centripetal than at the centrifugal pole. These eggs do not develop further (Fig. 14).

Immediately following the breakdown of the germinal vesicle, eggs were centrifuged until the stratification was complete (Figs. 1, 15). Upon being inseminated immediately after removal from the centrifuge tubes they show a striking difference in reaction from the uncentrifuged eggs. Within 60 seconds after insemination a fertilization membrane begins to separate from the heavy pole of the egg. During the next few minutes the perivitelline space widens asymmetrically, so that its greatest width is at the centrifugal pole. At the same time, thickening and hardening of the fertilization membrane is taking place at the centrifugal pole. At the centripetal pole of the egg there is usually no membrane separation. Consequently at this pole there is no perivitelline space, and thickening of the vitelline membrane on the egg surface does not occur. In these cases we may say that there is no fertilization membrane at the centripetal pole. Occasionally a membrane may separate slightly from the centripetal pole. The perivitelline space in such cases is always very narrow. The degree of thickening of the fertilization membrane parallels the width of the perivitelline space. (At the surface of the unfertilized egg or egg fragments of *Asterias* the vitelline membrane is too thin to be readily visible. See also Chambers, 1921*b*, and Hobson, 1927.) From the centrifugal to the centripetal pole of these strongly centrifuged eggs there is usually a gradual transition from the normal fertilization membrane to the completely unchanged vitelline membrane.

Eggs fragmented inside of the jelly-hull (Figs. 2, 3, 4), upon immediate fertilization subsequent to centrifuging, show marked differences in the membrane separation of the two fragments. The centrif-

ugal heavy fragment separates a fertilization membrane on all sides, giving rise to a perivitelline space somewhat narrower than in the normal egg. The fertilization membrane of this heavy fragment subsequently thickens and hardens in about the same time as is required for a normal fertilization membrane. The centripetal light fragment, presumably entered by a different sperm, usually separates a fertilization membrane from only a portion of its centrifugal pole. Here a very narrow perivitelline space results, accompanied by but slight thickening of the fertilization membrane. The surface of the centripetal pole of the light fragment (the vitelline membrane) is continuous with the slightly thickened fertilization membrane of the heavier pole of this fragment (Figs. 6, 7, 8).

Once the fertilization membrane has hardened there is no further change in its appearance. Subsequent cleavage of the egg or egg fragments serves to demonstrate more completely the differences between the membranes at the two poles of the centrifuged eggs. Figures 11 and 12 demonstrate two points: first, that the fertilization membrane of the centrifugal heavy fragment keeps the blastomeres together, while the lack of a fertilization membrane about the centripetal fragment permits them to separate; and second, that the hyaline plasma membrane of the starfish egg (as Chambers, 1930, has pointed out) is not sufficiently strong to keep the blastomeres together when the fertilization membrane is lacking.

Although the jelly-hull is thinnest at the centripetal pole of the fragmented egg (Fig. 3), this cannot be related to the difference in membrane separation, since a membrane separates from the centripetal pole of the heavy fragment, where the heavy fragment is in contact only with the light fragment, and not with the jelly-hull. There is, of course, much other evidence (see E. N. Harvey, 1914, Hobson, 1927) to demonstrate that the jelly (*zona pellucida* of Gray and Hobson) is unnecessary for the separation of a fertilization membrane.

In certain fragmented eggs a small spherical globule remains between the light and heavy fragment, This is not the first polar body, but is a protoplasmic globule included by the strand of the vitelline membrane connecting the two egg fragments. Since the vitelline membrane is not readily visible on the surface of the egg fragments, this, supplementary to certain microdissection experiments of Cham-

PLATE I

All photomicrographs are of living *Asterias* eggs, at a magnification of about 140 diameters. The centripetal pole is uppermost in each case. The top row of figures is of unfertilized eggs, the second row shows the results of fertilizing such eggs; and the third row, the cleavage of eggs similar to those of the upper rows.

Fig. 1.—Egg stratified by centrifuging 12 minutes, 10 minutes after entering sea water; photographed 2 minutes later.

Fig. 2.—Egg fragmented by centrifuging 10 minutes, 30 minutes after entering sea water; photographed 5 minutes later.

Fig. 3.—Egg fragmented by centrifuging 11 minutes, 40 minutes after entering sea water; photographed 6½ minutes later. The jelly has been outlined by placing the egg in dilute Janus Green.

Fig. 4.—Egg fragmented by centrifuging 11 minutes, 40 minutes after entering sea water; photographed 3 minutes later. The centripetal fragment is pulling out through the jelly.

Fig. 5.—Egg centrifuged 12 minutes, 64 minutes after entering sea water; inseminated immediately after centrifuging and photographed 53 minutes later. No fertilization membrane is visible at the centripetal pole.

Fig. 6.—Egg centrifuged 9 minutes, 43 minutes after entering sea water; inseminated immediately; photographed 71 minutes after insemination.

Fig. 7.—Egg centrifuged 19 minutes, 45 minutes after entering sea water; inseminated 1 minute later; photographed 20 minutes after insemination.

Fig. 8.—Egg centrifuged 17 minutes, 54 minutes after entering sea water; inseminated 1 minute later; photographed 27 minutes after insemination.

Fig. 9.—Egg centrifuged 9 minutes, 43 minutes after entering sea water; inseminated immediately; photographed 97 minutes later.

Fig. 10.—Egg centrifuged 12 minutes, 64 minutes after entering sea water; inseminated immediately; photographed 258 minutes later.

Fig. 11.—Egg centrifuged 12 minutes, 64 minutes after entering sea water; inseminated immediately; photographed 294 minutes later. The centripetal blastomeres are held together only by cell bridges.

Fig. 12.—Egg centrifuged 13¼ minutes, 73 minutes after entering sea water; inseminated 1¾ minutes later; photographed 340 minutes after insemination.

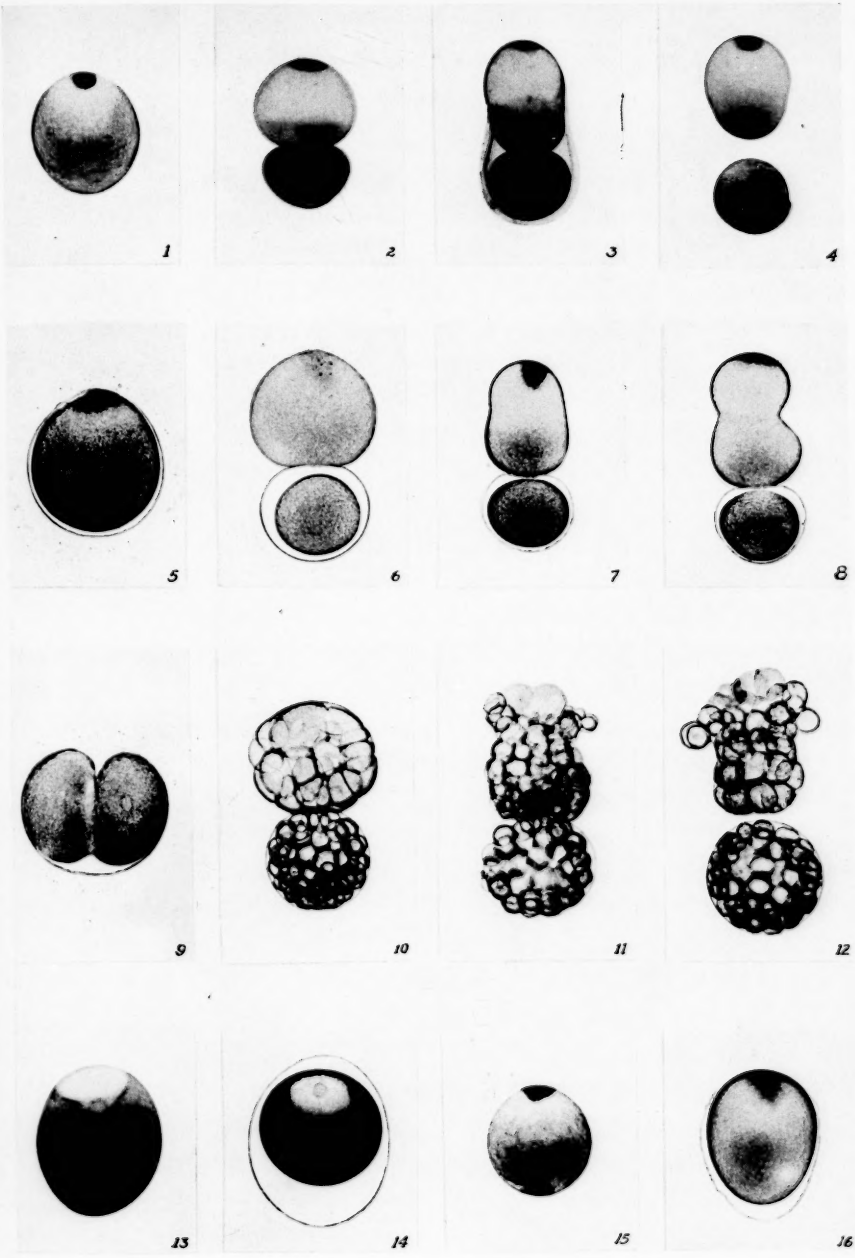
Fig. 13.—Egg centrifuged 18 minutes, 65 minutes after entering sea water. The germinal vesicle is intact, the nucleolus distending it centrifugally.

Fig. 14.—The fertilization membrane of an egg similar to Fig. 13. Centrifuged 12 minutes, 64 minutes after entering sea water; inseminated immediately after centrifuging and photographed 294 minutes later. The fertilization membrane is slightly thinner at the centripetal pole.

Fig. 15.—Egg centrifuged 12 minutes, 10 minutes after entering sea water; photographed 2 minutes later.

Fig. 16.—The fertilization membrane of an egg similar to Fig. 15. Egg centrifuged 9 minutes, 43 minutes after entering sea water; inseminated 1 minute after centrifuging and photographed 62 minutes later. The first and second polar bodies are visible.

PLATE I



bers (1921*b*) and cutting experiments of Whitaker (1931), constitutes additional evidence for the existence of the vitelline membrane on the surface of the unfertilized starfish egg. In cases where the egg fragments are pulled farther apart by the centrifugal force, the connecting strand of the vitelline membrane is broken. If this strand should remain intact, upon insemination the fertilization membrane of the heavy fragment will be found to be continuous with the thin fertilization membrane of the lower pole of the light fragment. If the clear globule is present it will be included in the fertilization membrane as if it were a polar body. This constitutes some slight additional evidence (see Chambers, 1921*b*, in particular) that the fertilization membrane originates through changes in the pre-existing vitelline membrane.

DISCUSSION

Two separate phenomena, which are perhaps very closely related, may be distinguished as the result of the fertilization of centrifuged eggs. First, the perivitelline space is widest at the centrifugal pole; and second, the fertilization membrane is thickest at this pole. The difference in the width of the perivitelline space is probably not due to injury of the vitelline membrane, since the polar difference is always the same. The isotonic sucrose at the bottom of the centrifuge tube probably has no effect in bringing this about, since it is mixed with a small quantity of sea water, and that pole of the egg farthest removed from the sucrose gives the most abnormal fertilization reaction.

Chambers (1921*a*, 1921*b*) and Just (1923) have pointed out that endoplasmic spheres or buds do not separate a fertilization membrane. Possibly there is a difference in density between endoplasm and ectoplasm, resulting in a partial separation of these under prolonged centrifugal force. Lindahl (1932, Fig. 2) has shown that such a separation (or centripetal stretching of the cortex) may take place in the centrifuged egg of *Sphaerechinus granularis*.

At present it is not possible to give an adequate explanation of these phenomena.

In the centrifuged and fragmented eggs of *Arbacia punctulata* E. B. Harvey (1932) has shown that the hyaline plasma layer of the granular quarter (derived from the colorless centripetal half-egg) is much thicker than that of the clear quarter, and also that the fer-

tilization membrane of the clear quarter usually breaks after several cleavages of the egg fragment. Taylor (1931), by extremely prolonged centrifuging of the egg of *Urechis caupo* in the germinal vesicle stage, has obtained, upon subsequent fertilization, a much thicker fertilization membrane at the centrifugal pole.

SUMMARY

1. The zones of stratification of the strongly centrifuged and of the fragmented eggs of *Asterias* have been described.

2. Eggs fertilized after prolonged centrifuging at a period when they are easily stratified fail to give a normal fertilization reaction at the centripetal pole.

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THE DIFFERENTIATING CAPACITY OF VARIOUS REGIONS OF THE HEART RUDIMENT OF THE CHICK AS STUDIED IN CHORIO-ALLANTOIC GRAFTS[1]

(Three figures)

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IT IS the purpose of the present paper to examine by means of the chorio-allantoic graft the capacity for differentiation of the heart rudiments of the chick embryo. More specifically, it is to deal with the extent to which the heart rudiment at various stages during the coalescence of the right and left omphalomesenteric veins is self-differentiating or is an equipotential system. Verocay's (1905) description of the famous hen with seven hearts and Graper's (1907) experiments in which two complete hearts were obtained as a result of preventing the union of the omphalomesenteric veins, suggest that in the chick the heart rudiments are equipotential systems. That the amphibian heart exhibits such properties in its development has been shown experimentally by Ekman (1924, 1929), Stöhr (1924*a*, 1924*b*, 1925, 1927), Copenhaver (1926), and Goerttler (1928). In making the present analysis the procedure was as follows. First, the whole rudiment was grafted to determine its capacity to form all four divisions of the heart independently of specific vascular characteristics, coelom relations, etc. Second, pieces from different regions of the heart rudiments were tested to ascertain whether (1) each has the power to form a whole heart and (2) the sinus or "pacemaker" end of the heart rudiment differs in its developmental potency from pieces of more anterior levels. Third, the liver-forming potencies of the entoderm associated with the sinus end of the heart and diverging omphalomesenteric veins were tested.

MATERIAL AND METHOD

The heart primordia transplanted in these experiments were taken from chick embryos ranging from eight to twenty somite stages.

[1] It is a pleasure to express here my sincere thanks to Dr. B. H. Willier for suggesting the problem and for helpful advice throughout the course of the work. An abstract of the chief results given in this paper was published previously (Kumé, 1931).

Two types of transplants were made. First, the whole heart primordium including right and left omphalomesenteric veins was isolated and transplanted to the chorio-allantoic membrane in order to test its self-differentiating capacity. The entoderm near the anterior intestinal portal was removed as carefully as possible, but due to the intimate relation between them there is no doubt but that some remained attached and was transplanted with the heart tissue. Second, for an examination of equipotentiality, the heart primordium was divided into four pieces, namely, the anterior and posterior middle pieces, and the right and left omphalomesenteric ones. Figure 1 shows the manner in which the cuts were made. This separation into four pieces was adopted for each stage except for those from seventeen to twenty somites. By this time the omphalomesenteric pieces become too short to separate and transplant, as a result of concrescence, so that the primordium was now separated into anterior and posterior pieces along the future limiting line between ventricular and sino-atrial divisions. Each piece thus isolated was transplanted to a host embryo, or a pair of adjoining pieces was grafted to separate places on the same host.

As there is no clear demarcation between heart components in early stages of development, it is difficult to ascertain exactly the prospective value of each piece thus isolated; but according to Lillie (1908, p. 200), the most posterior division of the heart (the sinus venosus) is established by concrescence between the stages of twelve and eighteen somites. On the basis of his description the right and left omphalomesenteric pieces between the stages of twelve and eighteen somites may represent the future sinus venosus; the posterior middle one may be the prospective atrium in early stages, but later becomes sinus and atrium, and the anterior middle one may have a prospective value of bulbus and ventricle. No clue is found to determine the value of pieces in stages younger than twelve somites. Probably the omphalomesenteric pieces are the future sinus and atrium, and the anterior and posterior middle ones the future bulbus and ventricle.

The method of grafting to the chorio-allantoic membrane was essentially the same as that described by Willier (1924). After re-

maining eight or nine days on the host membrane the grafts were removed and fixed in Bouin's fluid. They were sectioned at 6 μ and stained with Heidenhain's iron haematoxylin. In cases in which the

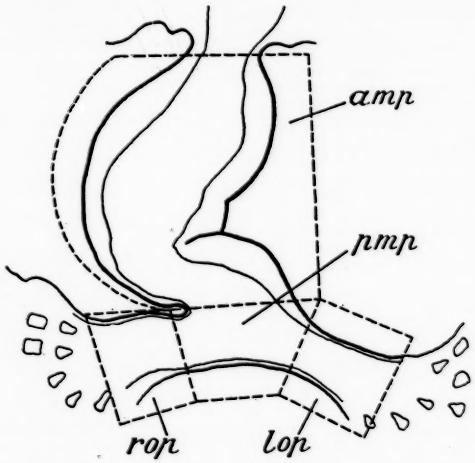

FIG. 1.—Diagram of a heart of a chick embryo of thirteen somites, showing the method of separating the whole primordium into four pieces: *amp*, anterior middle piece; *pmp*, posterior middle piece; *rop*, right omphalomesenteric piece; *lop*, left omphalomesenteric piece.

hearts were found to be pulsating when removed from the host, the rate of pulsation was taken in 0.9 per cent NaCl solution warmed to temperature of 39° C.

EXPERIMENTAL RESULTS

Grafts of the whole heart primordium.—A total of sixteen grafts of whole heart rudiments of embryos ranging from eight to twenty somites has been examined. The results are given in the accompanying summary, Table I. In eleven grafts out of sixteen the heart was pulsating regularly; in one graft it was found twitching irregularly, while in the remaining four grafts no pulsation was recognized.

Owing to the technical difficulty of removing completely the entoderm from the heart tissue, it was sometimes included in the transplants. Thus, in nine grafts, the gut epithelium is differentiated as

TABLE I

TABULAR SUMMARY OF RESULTS

Type of Isolation	Age of Donor in Somites	No. of Trials	No. of Grafts Obtained and Examined	Pulsation Exhibited	Lumen Present	Grades of Muscle Differentiation				Liver	Gut
						I	II	III	IV		
Entire rudiment	8		1	0	0	0	1	0	0	0	0
	9		1	1	0	0	1	0	0	1?	1
	10		2	2	0	0	2	0	0	0	2
	11		2	2	1	0	1	0	1	0	2
	12		3	2	1	0	0	1	2	0	3
	13		3	2	1	0	2	0	1	0	0
	14		1	1	1	0	1	0	0	0	1
	17		2	1	1	0	2	0	0	0	0
	20		1	1	0	0	2	0	0	0	0
Right omphalomesenteric portion	8	5	1	0	0	0	1	0	0	0	1
	9	6	2	1	0	0	2	0	0	0	2
	10	7	3	2	1	0	3	0	0	1	2
	11	6	3	3	2	0	3	0	0	1	2
	12	7	2	2	2	0	2	0	0	0	2
	13	5	2	2	1	0	2	0	0	1	2
	14	4	3	3	3	1	4	0	0	1	2
	15	4	1	1	1	0	1	0	0	1	1
	16	5	3	3	3	0	4	0	0	3	3
Left omphalomesenteric portion	8	5	4	3	0	0	3	1	0	3	3
	9	5	4	1	0	0	2	2	0	0	3
	10	4	3	1	0	0	2	0	1	0	3
	11	4	0	0	0	0	0	0	0	0	0
	12	5	2	2	2	0	2	0	0	0	2
	13	3	2	2	0	0	2	0	0	0	2
	14	2	1	1	0	0	2	0	0	1	1
	15	3	0	0	0	0	0	0	0	0	0
	16	5	0	0	0	0	0	0	0	0	0
Posterior middle portion	8	5	0	0	0	0	0	0	0	0	0
	9	10	2	0	0	0	0	2	0	0	1
	10	6	2	1	1	0	2	0	0	0	1
	11	8	5	3	0	0	3	2	0	0	2
	12	4	2	1	0	0	1	0	1	0	0
	13	5	2	1	0	0	2	0	0	1	2
	14	3	3	2	1	0	3	0	0	1	1
	15	5	4	4	4	0	4	0	0	2	4
	16	5	3	3	3	1	3	0	0	2	2
	17	3	2	1	1	0	2	0	0	0	2
	18	3	1	1	1	0	1	0	0	1	1
	19	6	6	5	3	2	3	0	1	1	4
	20	2	2	2	0	0	2	0	0	1	1

TABLE I—*Continued*

Type of Isolation	Age of Donor in Somites	No. of Trials	No. of Grafts Obtained and Examined	Pulsation Exhibited	Lumen Present	Grades of Muscle Differentiation				Liver	Gut
						I	II	III	IV		
Anterior middle portion	8	4	0	0	0	0	0	0	0	0	0
	9	8	0	0	0	0	0	0	0	0	0
	10	5	0	0	0	0	0	0	0	0	0
	11	6	0	0	0	0	0	0	0	0	0
	12	8	0	0	0	0	0	0	0	0	0
	13	8	1	0	0	0	0	1	0	0	0
	14	3	0	0	0	0	0	0	0	0	0
	15	3	1	0	0	0	0	1	0	0	0
	16	3	1	0	0	0	0	1	0	0	0
	17	1	0	0	0	0	0	0	0	0	0
	18	4	0	0	0	0	0	0	0	0	0
	19	2	2	1	0	0	1	0	1	0	0
	20	3	1	0	0	0	0	0	1	0	0

a small vesicle or tubule near the heart. A small amount of liver-like tissue is found in one case only. Since no actual difference is recognized between the hearts in grafts with entoderm and those without it, a small amount of entodermal material apparently has no effect upon the development of the heart.

The heart in these grafts is essentially the same in character as described by Willier and Rawles (1931). Its form is usually ovoidal and its size is extremely small when compared with that of the normal embryo of the same age. Histologically it consists of a mass or masses of cardiac muscles, sometimes having a lumen or a number of lumens within them. Only in the best cases does it resemble very slightly the normal. However, even in such cases, the usual four divisions of the normal heart have not been identified. My results differ from those of Willier and Rawles in that the cardiac muscles seem to be less highly differentiated and the lumen within them less frequently developed. The direct connection of the lumen with the host blood vessels, as noted by Murray and Huxley (1925), and Willier and Rawles (1931), is found in only one case in my grafts. Since it is seen that the heart in these grafts of the whole rudiment does not differ in form and histological structure from that obtained

from grafts of parts of the heart rudiment, a detailed description of it will be summarized in another section.

Grafts of the right omphalomesenteric piece.—Out of forty-nine transplants in which the host remained alive, a total of twenty grafts was obtained. Upon removing the grafts from the membrane, the heart was found pulsating regularly in fourteen cases, twitching quite irregularly in three cases, with no sign of beating in the remaining three grafts. In two cases pulsation of two different rates was found at separate regions of the same heart. After sectioning it was revealed that these hearts consisted of two masses of cardiac muscle each in contact with the other. Which rate of pulsation belonged to which mass of cardiac muscle was not determined.

A summary of the results of histological examination and frequency of occurrence of heart grafts is given in Table I. As this table shows, the numbers of grafts obtained increases as the age of the donor increases, that is, from early stages, eight or nine somites, the percentage of grafts obtained is less than thirty-three, while from later stages, fourteen to sixteen somites (except fifteen somites), the percentage increases to more than sixty. Increasing tendencies for pulsation and lumen-formation are also seen in the table.

The histological nature, form, and size of the heart in the grafts are not different from that in the grafts of whole heart. In three cases the heart takes a pear shape, consisting of two masses of cardiac muscle each in contact with the other. It is interesting to observe that in one case each mass of cardiac muscle has a different grade of differentiation. Also, in one case, a lumen with a trabecular-like projection is found. Such a lumen resembles slightly the ventricular cavity of the normal heart (see Fig. 3).

The entoderm which is included in the transplants differentiates in most cases into gut epithelium. It is usually in the form of vesicles or tubules, sometimes surrounded by smooth muscle. Liver tissue is differentiated in eight grafts, all of which come from donors older than the ten-somite stage. Its relation to the heart is similar to that described by Willier and Rawles (1931).

Grafts of the left omphalomesenteric piece.—Out of thirty-six trials in which the host remained alive, sixteen grafts each containing heart tissue appeared, the results of the examination of which are

summarized in the table. In six grafts the heart was found pulsating regularly, in four cases it was twitching irregularly, while in the remaining six cases no pulsation was observed.

The frequency of the occurrence of these grafts containing heart tissue is interesting when compared with that from the right omphalomesenteric portion. While the number of "takes" increases as the age increases on the right, it decreases as the age of donors increases in the present series. As seen in the table, heart grafts were obtained the most frequently in early stages, eight or nine somites, at which time they appear with a frequency of eighty per cent. However, there is a gradual decrease which at last comes to zero at the fifteen- to sixteen-somite stage. A similar tendency is also found for pulsation. The lumen appears less frequently here than in grafts from the right piece.

Gut epithelium differentiates as a vesicle or tubule in most cases. Liver develops in three grafts from the eight-somite stage and in one graft from a fourteen-somite embryo. This tissue occurs more frequently in the grafts obtained from the right omphalomesenteric portion (see table).

Grafts of the posterior middle piece.—A total of thirty-four grafts obtained from donors ranging from the eight- to the twenty-somite stage were obtained out of sixty-five transplants in which the host remained alive. Among thirty-four grafts thus obtained, heart was found pulsating regularly in twenty-three cases, twitching irregularly in one case, and the remaining ten cases showed no sign of pulsation. Summarized results are shown in the table.

From the eight-somite stage no graft was obtained; from the nine-somite stage two grafts out of ten transplants were obtained, each containing heart tissue. These hearts both proved to be quite small and were not pulsating. From the ten-somite stage two grafts out of six transplants differentiated heart tissue; one of these was pulsating weakly at the time of its removal. These results indicate that the posterior middle piece at these stages has no capacity or perhaps only a weak capacity for the differentiation of cardiac muscle. After eleven somites the frequency of heart grafts gradually increases and at nineteen to twenty somites grafts showing cardiac

muscles are obtained from every transplant. The same increasing tendency is also seen in the occurrence of pulsation and lumen.

Gut epithelium is found in most cases to be differentiated as a vesicle or tubule. It has in some cases a covering of smooth muscle. Liver has not developed from transplants made earlier than thirteen somites. This indicates that the entoderm included in the present grafts has no capacity to produce the liver before the thirteen-somite stage. The relationship between heart, liver, and gut is the same as mentioned before.

Grafts of the anterior middle piece.—As this portion represents the prospective bulbus and ventricle which are the most muscular divisions of the normal heart, high muscular differentiation was at first expected from the transplants. However, it appears that this piece has no capacity, or only a very weak capacity to differentiate into cardiac muscles on the chorio-allantoic membrane. Actually, out of fifty-eight transplants which remained eight or nine days on the living hosts, only six grafts were obtained. All of these grafts contained cardiac muscle. One was beating regularly, but the remaining five showed no sign of pulsation. Thinking that the heart tissue might have formed a graft which did not exist for 8 or 9 days, four more transplants were examined at the third day, three at the fourth day, and one at the fifth day. But neither muscular differentiation nor degenerating muscles were found in the membrane after sectioning; even the transplants were not identified as such. Only the sections showed that something had occurred in the membrane, since besides a thickening, some whorl-like arrangement of the connective tissue elements was observed under the epithelium.

As seen in the table, the heart-grafts occur only from stages of thirteen somites and later. These hearts are small in size and their muscles appear to be less well differentiated.

Form and structure of the heart.—Since the hearts in the grafts are consistent in exhibiting the same structure in spite of their origin, they will be considered as a whole. A total of ninety-two grafts, each containing cardiac tissue, has been examined; sixteen derived from transplants of the whole heart rudiment, twenty from the right omphalomesenteric piece, sixteen from the left omphalomesenteric

piece, thirty-four from the posterior middle piece, and six from the anterior middle one.

In the majority of cases the heart of the graft takes an ovoidal form with smooth outline (see Fig. 2). But its surface may become somewhat irregular when a lumen is present (see Fig. 3). It is some-

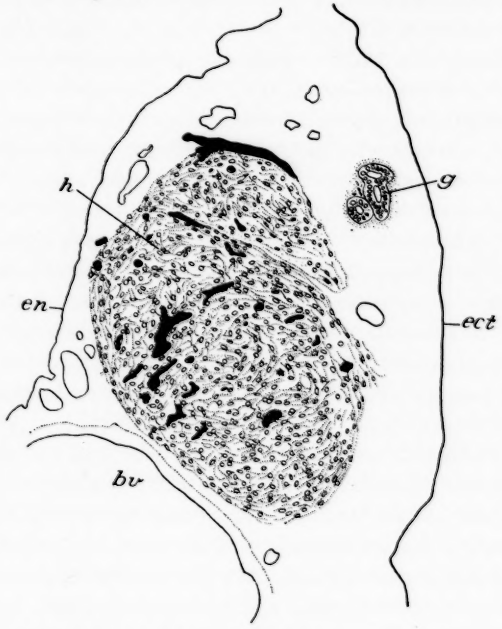

FIG. 2.—A somewhat schematic drawing illustrating the form, arrangement of cardiac muscle, and distribution of capillaries in a so-called heart obtained from a graft of the posterior middle piece of an eleven-somite embryo. *bv*, blood vessel; *ect* and *en*, ectoderm and entoderm of the chorio-allantoic membrane; *g*, gut epithelium; *h*, heart. (Initial ×150).

times pear-shaped, as if two masses of cardiac muscles had come together. Smaller masses of cardiac muscle take a round form usually; or, rarely, they appear elongated. The long axis of the heart lies parallel to the surface of the chorio-allantoic membrane, in spite of its form; but it may lie somewhat obliquely when other tissues such as gut and liver, lymph vesicles, or large blood vessels

of the membrane are in proximity. Even in cases in which the heart is elongated, its direction is nearly always parallel to the surface of the membrane. In size, the hearts range from only a trace of cardiac muscle to a mass of considerable size: the largest one measured 1125 μ in long axis, 675 μ in short axis and extended over 242 sections (1452 μ). This is small in size when compared with the normal heart of the same age.

Histologically the heart consists of a mass or masses of cardiac muscle which is embedded in the connective tissue of the chorio-allantoic membrane. Although there is no surface covering like an epicardium, outer muscles run parallel along its surface. Usually the heart consists of a single mass of cardiac muscle, but the larger ones sometimes consist of two or three masses each in contact with the other. In the latter cases each mass has its muscles arranged just like a single one (Fig. 2). In some instances the masses may have a different appearance from one another owing to a different density or grade of differentiation of the muscles, or to a different density of distribution of capillaries among the muscles. In two cases different rates of pulsation were found in the same heart. After sectioning, it was revealed in both that the heart consisted of two muscle masses. Although it was not determined which of the masses had a particular rate of pulsation, it is of interest to observe that in one case one of the masses was made up of muscles differentiated quite highly, whereas the muscles of the other one were of the type of those most often seen in these grafts. The cardiac muscles forming a mass are in most cases compact and form a syncytium. In rare cases they are scattered within a certain ovoidal area among the connective tissue elements of the membrane.

As to the differentiation of the cardiac muscle, it reaches in the majority of cases a grade, designated as Grade II in the table, in which the muscle fibers elongate and make a rather complicated bundle, but the striations of myofibriles are not differentiated so clearly. This grade of cardiac muscle does not seem to have differentiated as far as that described and figured by Willier and Rawles (1931, Fig. 4). The highly differentiated muscle, Grade I of table, comparable to those of the aforementioned authors is found in my grafts in four cases only. Other types of cardiac muscle differentia-

tion to be found in these grafts are "mesenchymatic" and "vacuolated" types, Grades III and IV respectively. Muscles of Grade III appear to be still less differentiated than Grades I and II. Their cell shape is star-like and the processes anastomose. Mitotic figures are found quite often among them. Muscles of Grade IV type are very peculiar and were first thought not to be heart muscles, but judging from the fact that some of these were pulsating while living, there is not much doubt as to their identity. Their nuclei are generally small in size and their sarcoplasm is somewhat vacuolated.

In about one-third of the cases the heart has a lumen or a number of lumens containing blood corpuscles. The number of cases in which the lumen appears seems to be less in my grafts than in those of Willier and Rawles, where it appears in the majority of cases. This difference of occurrence may be attributed to the presence of other tissues. When a lumen is present, it takes a central position in the heart, in a majority of cases, but sometimes it is eccentrically placed. A number of small lumens may be distributed throughout the heart-muscle tissue.

The direct connection of the lumen with the larger blood vessels of the chorio-allantoic membrane is found only in two or three of my cases. This fact is also in contrast with that noted by Willier and Rawles. In their grafts the lumen was found in most cases to be connected at one or both ends with the larger host vessels. Perhaps this difference may also be attributed to the presence of other tissues in the graft. In some cases in which a large lumen develops trabecular-like projections of it are seen. The most typical one of these is shown in Figure 3. This trabecular-like projection is probably the remanent of the walls of smaller lumens which have fused.

Regardless of whether or not the lumen is present, the heart is vascularized by a dense network of capillaries which come in from the surrounding connective tissue of the membrane. The distribution of these capillaries among the heart tissue is far denser than that among the surrounding connective tissue.

The heart in 64 cases was found pulsating at the time of removal from the chorio-allantoic membrane (see Dantschakoff and Gagarin, 1929). In the majority the pulsation was strong and regular. Nine cases showed irregular twitching. When the heart was found beating

regularly, its rate was counted roughly. The rate of pulsation was found to be exceedingly variable. Some gave only 20 pulsations per minute, while others gave as many as 100.in the same time. The average rate was about 71 beats per minute. Records were made at 39° C. It is of interest to observe that strong regular pulsation occurs regardless of whether or not liver tissue is present in the graft.

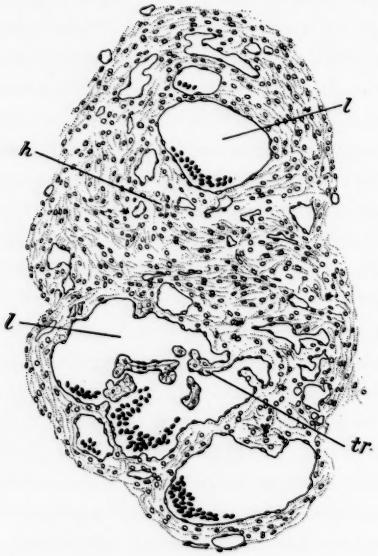

FIG. 3.—A somewhat schematic drawing illustrating the form and histology of a so-called heart from a transplant of the right omphalomesenteric piece. Note the lumen with trabecular-like projection (*tr*), *h*, heart; *l*, lumen. (Initial ×150).

The liver and its relation to the heart.—In a total of twenty-two grafts the liver is differentiated; eight cases from grafts of the right omphalomesenteric piece, four cases from those of the corresponding left one, nine cases from the posterior middle piece, and a questionable one from a transplant of the whole-heart rudiment.

The liver in the grafts is histologically typical, consisting of hepatic cords and sinusoids. Its shape is quite irregular and its size ranges from a few hepatic cords to a size of 600 μ in long axis, 330 μ in short axis, and covering 80 sections (480 μ). The relationship be-

tween heart and liver in the grafts is the same described by Willier and Rawles (1931). In the twenty-two grafts in which liver has developed it is either in contact (seven cases) or in close association with the heart tissue. When gut is present, liver is differentiated from the gut toward the heart (eight cases). In two cases in which no gut has developed, liver is situated a short distance from the heart. Further, it is observed in my cases that the liver develops only in the grafts in which the heart was found pulsating. This relationship may be useful for further analysis of correlative development.

DISCUSSION

Differentiation of the entire heart rudiment and the regulation of form in the grafts.—The entire heart rudiment, ranging from eight to twenty somite stages, when isolated and transplanted to the chorio-allantoic membrane, differentiates as a mass or masses of cardiac muscle with or without a lumen. Of the sixteen grafts which have been examined, eleven showed hearts of only a single mass of cardiac muscles; in the remaining five cases the heart consisted of two masses of cardiac muscles in contact or partly fused. There is no reason for regarding this mass as cardiac muscle as representing some particular component of the normal heart.

These results are in contrast to those obtained by Dantschakoff and Gagarin (1929) who report that the heart of a chick embryo incubated from three to four days continues to develop rather normally on the chorio-allantoic membrane. The difference in our results can hardly be explained on the basis of a difference in stage used or in the duration of the graft on the membrane.

That the heart differentiates in the grafts into such a mass or masses of cardiac muscle is not a feature peculiar to the grafts of the whole-heart primordia, but is equally characteristic of the grafts of its parts. Such behavior on the chorio-allantoic membrane may be due to the abnormal conditions of the graft. While the normal heart develops within a pericardium, with the constant inner pressure of the blood stream, the heart in the grafts develops within the connective tissue of the chorio-allantoic membrane with no regular blood-stream pressure, and in all probability under very abnormal strains and stresses due to tensions in the membrane. The importance of

blood stream for the normal development of the heart is especially emphasized by Stöhr (1925, 1931) who found that the heart developed poorly, especially the ventricle, if the quantity of the blood stream was lessened, or its viscosity decreased. That the presence of connective tissue may hinder in some way the development of the heart in the grafts is not inconceivable. Further, the fact that the large mass of cardiac muscle in the grafts generally takes an ovoidal form, its long axis being in most cases directly parallel to the surface of the membrane, may indicate that the growing heart is affected by an outer pressure due to tension in the membrane.

Lumen development occurs in the grafts of the whole-heart rudiment as well as in those of its parts. As the table shows, lumens appear more frequently in hearts obtained from older stages than from younger ones. This fact indicates that the heart acquires a stronger capacity for production of lumens as the age increases. I find that the lumen appears less frequently in those cases in which none or only a small amount of other tissues was included in the transplants. The presence of large amounts of various other tissues may possibly have some effect on lumen formation. The fact that a direct connection of the lumen with the host vessels is found quite rarely in my grafts shows that its formation occurs independently of the blood stream. It is probable from these facts that the factor for the development of the lumen is intrinsic in the cardiac muscle or even in the endothelium itself.

The cardiac muscle in my grafts does not seem to be differentiated histologically as highly as that described by Willier and Rawles (1931); neither do I find the close association with ganglionic tissue which they mention.

Differentiating capacity of the various regions of the heart primordium.—Results of transplanting the four separated pieces of the heart primordium show that the pieces producing cardiac muscle most frequently are the right and left omphalomesenteric pieces and the posterior middle piece, while the piece representing the future bulbus and ventricle differentiates either none or only a small amount of cardiac muscle. These results are contrary to expectation, since the bulbus and ventricular divisions are the most muscular parts of the heart. The cause for this absence of differentiation or

weak differentiation of cardiac muscle from the piece representing the future bulbus and ventricle is puzzling.

As far as my results are concerned, the whole epimyocardium has the potency to produce cardiac muscle. The fact that the left and right omphalomesenteric pieces and the posterior middle piece give cardiac muscle more frequently and also give a higher grade of differentiation than the anterior middle portion shows that there are regional differences of some sort along the heart tube. The posterior region of the heart (the sino-atrial region) first has the power to form cardiac muscle, and this power proceeds gradually forward to the bulbo-ventricular region as development progresses. If this is true, it is to be expected that the piece representing the future bulbus-ventricle will produce cardiac muscle more frequently at some later stage. Further investigation is necessary to settle this point. It is of interest to note here that the region which first has the potency of forming cardiac tissue becomes the center of pulsation and has the highest metabolic activity of any level of the heart tube (Hyman, 1927). According to Copenhaver (1930), this region in addition acts as a pace-maker for the heart.

Relation between heart and liver in the grafts.—It is next proposed to discuss briefly some problems concerning the relation of liver substance to the developing heart in the grafts (cf. Stöhr 1924*a* or *b* and 1929). He attributes the failure of heart (*Bombynator*) to differentiate when transplanted to the peritoneal cavity, to the absence of liver tissue from the transplant. Although it is not clear which region of the heart was included in his transplants, his results are comparable to those of the grafts from the anterior middle piece of my cases.

Willier and Rawles find in grafts of the whole blastoderms that the liver develops only in the grafts in which the heart differentiates, while the heart develops independently of the liver. They suggest that there is some correlative influence there. Also, they find no difference in the nature of heart tissue in the grafts in which the liver is found in contact with the heart as compared with the hearts which developed in the absence of liver or not in contact with liver. These results are corroborated by my cases. Moreover, I find that the liver develops only in grafts in which the heart is pulsating; yet the heart

pulsates in grafts in which the liver is absent. The presence or the absence of liver apparently has no effect on pulsation.

Bilaterality of heart and liver-forming potencies and their asymmetrical differences.—The fact that grafts from the posterior middle piece, which includes the central portion of the anterior intestinal portal, do not give liver before the thirteen-somite stage, while the grafts from the right and left omphalomesenteric pieces produce it earlier, shows that before this stage the power to produce liver is localized bilaterally in the entoderm included in the right and left omphalomesenteric pieces. (Cf. Willier, Rawles and Rudnick, 1931.)

Inspection of the table shows that there are some differences in the frequency of occurrence of both the liver and the heart in the grafts from the right omphalomesenteric piece when compared with those of the left one. The frequency of occurrence of the heart from the right omphalomesenteric piece gradually increases as the age increases; on the other hand, the grafts of the left one produce heart most frequently at the eight- or nine-somite stages; its frequency gradually decreases as the development progresses. Goerttler (1928) has reported asymmetrical differences regarding pulsation in the right and left heart primordia of Amphibian larvae. The part which has the greatest capacity for producing cardiac muscle within the left omphalomesenteric piece is asymmetrically localized as compared with the corresponding part in the right one. In other words, such a part in the left piece is localized much nearer to the posterior middle piece than the corresponding part in the right one, and as the heart is lengthened backward by the concrescence of the omphalomesenteric veins, such a part in the left piece shifts within the area of the posterior middle piece at an earlier time than that in the right piece. The asymmetrical difference found in the present grafts may have some connection with the difference in size of the left and right omphalomesenteric veins in the normal embryo; the former being usually larger than the latter.

Asymmetrical differences are found also as regards the frequency of occurrence of liver. Before the thirteen-somite stage the liver appears in the grafts from the left piece only at the eight-somite stage, while it appears in the grafts from the right one after the ten-somite stage. Although the number of my cases is small, the data suggest

that the right and left liver-forming areas, which may be localized near the part of the heart which has the strongest capacity to produce the cardiac muscles, are asymmetrically localized and are determined at different times. That is to say, the left liver-forming area is determined earlier than the right and localized much nearer to the central portion of the anterior intestinal portal.

SUMMARY

1. Chorio-allantoic grafts obtained from transplanting the entire heart rudiment as well as isolated pieces of the heart rudiment of chick embryos ranging in age from eight to twenty somites have been studied. Many of the grafts exhibited pulsation at the time of removal from the membrane. Histological examination of ninety-two grafts recovered shows that the heart rudiment differentiates as a mass or masses of cardiac muscle without any evidence of the usual four divisions. The form in most cases is ovoidal.

2. The anterior middle piece has no capacity to differentiate into cardiac muscle until the thirteen-somite stage. After this time it acquires a weak capacity to differentiate, producing a small "heart" with slight frequency.

3. Although it has little or no capacity to produce the cardiac muscles when grafted at an early stage (eight, nine, and ten somites), the posterior middle piece differentiates cardiac muscle more frequently as the age increases. A parallel fact is also seen in regard to pulsation.

4. In the grafts from the right omphalomesenteric piece, frequency of grafts containing heart tissue increases as the age increases (from 20 per cent at eight somites to 60 per cent at sixteen somites). On the contrary, in the grafts from the left piece, the frequency decreases as the development progresses (from 80 per cent at the eight-somite stage to zero at the fifteen- or sixteen-somite stage).

5. Liver is found only in the grafts which exhibited pulsation.

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STUDIES IN POPULATION PHYSIOLOGY. IV. SOME PHYSIOLOGICAL EFFECTS OF CONDITIONED FLOUR UPON TRIBOLIUM CONFUSUM DUVAL AND ITS POPULATIONS

(One figure)

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THE problem of the effect of environmental contamination or conditioning upon organisms and their populations is not a new one. It has long been recognized, for both water and terrestrial forms, that as animals and plants inhabit their environment they modify it in such a way that they are themselves frequently affected as a result of such modification. Many observations and some precise data are available on the nature of these effects. Sometimes the organisms and their populations are favorably influenced by this conditioning; frequently they are injuriously affected. Much of the general knowledge of this subject has been collected and presented by Allee (1931, 1934), and repetition here would seem unwarranted. To contribute further to this field, a study, part of which is presented here, is being made on the effects of self-environmental conditioning upon the flour-beetle *Tribolium confusum* and its populations. This form spends all its life-cycle in flour which may be collected and its effects upon the beetles assayed. This "conditioning" of the medium by *Tribolium* was long ago incidentally recognized by Chittenden (1896), who said, in discussing the flour-beetles as household pests, ". . . . but after the flour has been barreled or placed in bags and left unopened for any length of time the adult beetles make

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their appearance, and in due course the flour is ruined, for when the insects have time to propagate they soon convert the flour into a gray useless mass" (pp. 112–13). Stanley (1932, p. 634) also noted the possibility of conditioning when he stated, ". . . . They [*Tribolium*] require almost no care to rear, beyond seeing that the flour does not become filled with excreted waste." In a recent paper Park (1934*a*) showed that the effective reproductive rate of *Tribolium* living in conditioned flour was significantly lower than that of control beetles living in fresh flour. During the first ten days of the experiment the conditioned-flour beetles produced about one-third as many eggs as did the fresh-flour beetles, and after 60 days the experimental forms produced about one-ninth as many eggs. This was considered an effect of environmental conditioning upon the reproduction of the beetles and consequently their population size. In this previous paper, however, no attempt was made to analyze what physiological processes were affected, and it was explicitly pointed out that no knowledge was available as to the real factors in conditioned flour which might produce the biological effect demonstrated. The present paper is concerned with an analysis of some of the types of processes influenced by heavily conditioned flour and the significance of these effects from the viewpoint of the population as a whole. Researches as to the important biological and chemical factors of the conditioned flour responsible for the described physiological responses will be reserved for future treatment. The experimentation presented in this paper deals with the following aspects: first, the effect of conditioned flour on cannibalism; second, the effect of conditioned flour upon fecundity; third, conditioned flour and fertility; fourth, conditioned flour and the rate of metamorphosis; and, last, conditioned flour and the mortality of metamorphosis. It will be apparent that all of these processes are directly related to population growth, maintenance, and decline.

METHODS

Much has been said about the technique of handling *Tribolium* and this need not be repeated here. Suffice it to say that the adult beetles and all their immature stages may be readily separated from infected flour with counts made of their numbers. A recent survey

of the general biology of *Tribolium confusum*, which includes remarks on handling and culturing, has been made by Park (1934*b*) and may be consulted for further information about the organism. The conditioned flour used in all the experiments was taken from several heavily infested *Tribolium* stock cultures which had existed as concentrated cultures for about 100 days and had started their decline. The flour is thus defined in functional, biological terms. It should be emphasized that the experimental flour used in all the investigations reported in this paper is to be regarded as heavily conditioned (self-contaminated) since it was obtained from cultures which had well passed their asymptotic state. A whole range of new problems may be opened up when flour in intermediate stages of conditioning (i.e., flour taken from younger or less dense cultures) is used in similar experimentation. The control or fresh flour was an unbleached white flour ("Ceresota"). This flour was adopted since it had been used in past work (Park, 1932, 1933, 1934*a*) and since it was the original source of the conditioned flour. It is realized that a whole-wheat flour is probably a more favorable environment for *Tribolium* than patent flour since beetles have a somewhat higher rate of reproduction therein (Good, 1933), and probably the populations reach a higher asymptotic point in their growth. Norris (1933) has demonstrated facts for the flour moth *Ephestia kühniella* Z., which agree essentially with the foregoing. It is not considered a serious criticism of the present work, however, that unbleached patent flour has been used here because the beetles do live successfully in such an environment and, as some of the following biometric data show, reproduce and metamorphose without unusual or unexpected variation for randomly chosen samples. Furthermore, the beetles are found successfully inhabiting many grains in nature some of which are obviously less favorable than others. In addition, there is the very valid point that in the present experiments control and experimental flours always differ relative to each other, so that differential effects due to conditioning may be clearly demonstrable.

The experiments were run in darkened incubators at a constant temperature of $28 \pm \frac{1}{2}$°C., and a relative humidity between 40 and 50 per cent.

Recent evidence (Chapman and Baird, 1934) indicates that vio-

lent shaking or handling of *Tribolium* alters their fecundity. For this reason the adults were never sifted in any of the present experiments but gently removed from the infected flours with brush and spatula.

Since the *Tribolium* stocks used in these and past experiments (Park, 1932, 1933, 1934*a*) have been kept in the laboratory for several years, it was considered wise to have them re-examined to see if they were still pure and uncontaminated. Accordingly, generous samples chosen at random from three different *Tribolium confusum* cultures were sent to Dr. Harold Morrison, in charge of insect identification and classification, Bureau of Entomology, United States Department of Agriculture. Dr. Morrison kindly referred the specimens to Dr. E. A. Chapin of the same department, who examined each individual beetle from all three samples and found them all to be *Tribolium confusum* Duval.

Allee (1931) showed by recalculating Chapman's (1928) data, and Maclagan (1932) and Park (1932, 1933) have experimentally substantiated the fact, that for initial populations there appears to be an optimal concentration of beetles at which size the greatest population growth occurs. This concentration was shown to be smaller than the largest initial beetle seeding and larger than the smallest initial beetle seeding. Park emphasized that this was an effect apparent only in the first 11 days of culture growth, after which time it disappeared. Chapman and Baird (1934, pp. 302–3) have recently questioned the possibility of the existence of such a phenomenon for *Tribolium*. Since some of the points made by these authors, in this connection, appear invalid to the present writer, it seems advisable to deal briefly with them here.

If my interpretation of Chapman's remarks is correct, it appears that he criticizes the previous work on two scores: first, because the rates of oviposition obtained by Park differed from the more "constant" rates obtained by himself; and, second, that the variation in fecundity displayed by the *Tribolium* used by Park was greater than that obtained by Chapman. The temperature and humidity conditions were quite similar in the two investigations, although not identical. Chapman used a temperature of 27° C. and a relative humidity of 75 per cent; Park used a temperature of 28° C. and a relative

humidity of 40 per cent. Chapman's and Baird's own conclusion on the matter is:

> . . . . There may, therefore, be some doubt as to whether he [Park] was actually measuring the effects of the number of individuals present in the environment on oviposition, and whether his results may not have been greatly influenced by the variations between individual females [p. 303].

In the first place, there does not appear to be any real biological reason why the whole species *Tribolium confusum* Duval should have a constant rate of oviposition with coefficients of variability extremely small, or, as Chapman and Baird put it, ". . . . Its [*Tribolium*] biotic constants are sufficiently exact to warrant the use of mathematical formulae for calculating the potential trends of the population" (p. 303). In other words, even if environmental conditions were as similar as possible for both sets of experiments (Chapman's and Park's), why should the beetle populations of Park, most probably genetically different from those of Chapman and undoubtedly under certain different ecological conditions, give essentially the same "biotic constant" of reproduction as do the populations of Chapman? Probably the most important reason for the difference in expressed fecundity between the stocks of Chapman and Park is the fact that Chapman cultured his beetles on whole-wheat flour and Park used an unbleached patent flour. This whole question has been discussed in more detail elsewhere (Park, 1934*b*).

Chapman and Baird also criticize the results on the ground that extremes of egg-laying variation on the part of individual females could blot out the crowding effect previously shown since one can choose from the data individual females from the least crowded environment which have as high a fecundity rate as those in more favorably crowded cultures. It seems here, however, that Chapman ignores the statistical side of the question in dealing with Park's data, although he emphasizes this same point in considering his own material (p. 300). Chapman selects individual cases from the whole body of data to make his point and neglects the general trends as represented by the series taken as a whole. Park tested and recorded the statistical significance of the differences for the various populations and found that the most productive initial culture gave significantly more eggs and larvae than the others. This statistical test makes al-

lowances for the matter of individual variation and compares, for all cases, the rate of reproduction of one series with the other.

EXPERIMENTATION

CONDITIONED FLOUR AND CANNIBALISM

In earlier publications (Chapman, 1928; Stanley, 1932; Park, 1932, 1933) the importance of cannibalism in influencing *Tribolium* populations has been emphasized. Chapman has shown that the rate of egg consumption by adult beetles and larvae varies in direct proportion with the population concentration and concludes this to be a most important factor in explaining the population equilibrium reached by *Tribolium* cultures. Park (1933) obtained egg-eating rates for both male and female beetles and showed, at least within the limits of the specific experimental conditions used, that the two sexes ate eggs at statistically the same rate. Park (1934*b*) has described the general behavior of *Tribolium* in the process of eating its own eggs.

Since cannibalism is an important factor in *Tribolium* populations it seemed advisable to investigate the effect of conditioned flour on it. Consequently, experiments were set up to test this point. The controls consisted of 40 bottles each supplied with 32 gm. of fresh flour and each bottle containing 30 eggs randomly distributed throughout the medium. The experimental group was precisely similar to the control except that each bottle contained 32 gm. of conditioned flour in place of fresh flour. Previous to the start of the experiment, 80 male pupae had been obtained from a culture and divided into two groups of 40 each. Half of these pupae were allowed to emerge and live 15 days in fresh flour and the other half 15 days in conditioned flour. At the end of that time the males from fresh flour were introduced one per bottle into the fresh-flour series and the males from conditioned flour started in the conditioned-flour series. The experiment ran 9 days without interruption, and on the tenth day the flour was sifted and counts were made of the number of eggs remaining out of the original 30. After counting, each male was returned to his respective bottle—the experimentals to their same 32 gm. of conditioned flour and the controls to 32 gm. of renewed fresh flour, and again 30 eggs were supplied each culture. On the next

tenth day egg counts were made. This procedure was continued until five readings, or 50 days of experiment, had been obtained. The data are presented biometrically in Table I.

Probably the most striking difference between the control and experimental results is the fact that the fresh-flour males are consuming eggs essentially twice as rapidly as are the conditioned-flour beetles. The difference between the mean number of eggs eaten when compared with the probable error[2] of that difference shows the results to fall outside the realm of chance. At least, under the conditions tested, it seems safe to conclude that conditioned flour lowers the rate of

TABLE I

CANNIBALISM AND CONDITIONED FLOUR

	Total No. Eggs Eaten in 50 Days	Maximum No. Eggs Eaten by Any Single Male	Minimum No. Eggs Eaten by Any Single Male	Mean No. Eggs Eaten per Male per 50 Days	Standard Deviation	Coefficient of Variability (Per Cent)	n
Fresh-flour males.....	831	36	10	$20.7 \pm .62$	$5.9 \pm .43$	28.5 ± 2.15	40
Conditioned-flour males..	438	20	4	$10.9 \pm .44$	$4.2 \pm .31$	38.5 ± 2.91	40

Probability of the difference between means, $P < .0000$ (difference, $13 \times PE$)

egg consumption for adult *Tribolium*. The two coefficients of variability suggest that the relative egg-eating variation is greater for the conditioned-flour males than for the fresh-flour ones. This is not a too safe deduction, however, since the difference between the two C.V.'s is on the borderline of statistical significance ($P = .0686$). Exactly what physiological effect operates to cause the difference in the rate of egg-eating between males from conditioned and unconditioned media is not clear. It may be that conditioned flour lowers the general physiological level of the organism and thus reduces its food intake. On the other hand, the effect may be less of a biological one: the eggs possibly being harder to locate in conditioned flour; the beetles moving less therein, etc.

[2] Probable errors are used throughout this paper.

From the point of view of interpreting population trends this cannibalism effect would seem of slight importance in explaining population decline. A lowered cannibalistic rate would go hand in hand with aging and conditioning of the environment, and would obviously not favor a decrease in numbers. Presumably, but not proved, this effect, if it existed at all, would be of little significance in young actively growing cultures in which the flour was in a comparatively fresh state.

CONDITIONED FLOUR AND FECUNDITY

In considering physiological processes of importance in the study of populations, fecundity and fertility must be emphasized. Pearl (1927) points out that the two primary biological variables involved in all population trends are the force of natality, measured by the birth-rate, and the force of mortality as measured by the death-rate. Natality *per se* will be closely associated with the physiology of fecundity and fertility. The relation of conditioned flour to the latter processes will be experimentally considered.

CONDITIONED FLOUR AND VIRGIN-FEMALE FECUNDITY

Experiments were designed to test the effect of conditioned flour upon the egg production of virgin-female beetles. Park (1933) showed that *Tribolium* females which had never experienced copulation were able to produce infertile eggs. These eggs were laid at a lower rate, however, than those oviposited by fecundated females. When investigating conditioned-flour effects upon virgin-female oviposition, one is measuring solely fecundity as fertility considerations are excluded.

An experiment was set up so that the oviposition rates of virgin females in fresh and conditioned flour could be compared. The control consisted of 100 gm. of fresh flour plus 25 virgin-female *Tribolium*. The experiment consisted of 100 gm. of conditioned flour plus 25 virgin females. The beetles used were randomly selected as pupae from the same stock. Half of the pupae were placed in conditioned and half in fresh flour and allowed to remain in these environments for 15 days. At the end of that time the fresh-flour females were placed in the 100-gm. fresh-flour culture and the conditioned-flour females in the conditioned flour medium. The experiment was run

45 days, with egg counts made every 5 days. After counting, the females were returned to their respective environments and the eggs destroyed. The results are presented in Table II. The striking conclusion is immediately apparent that the virgin females from fresh flour produced three times as many eggs as did the conditioned-flour beetles. This difference, as can be seen from the probability figure of Table II, is a very significant one. It seems clear, at least in this case,

TABLE II

OVIPOSITION RATES FOR FRESH AND CONDITIONED-FLOUR VIRGIN FEMALES

Time (in Days)	Fresh-Flour Females		Conditioned-Flour Females	
	No. Eggs per 25 Virgin Females	Per Cent of Total No.	No. Eggs per 25 Virgin Females	Per Cent of Total No.
5	54	11.92	18	11.84
10	50	11.04	17	11.18
15	32	7.06	12	7.89
20	26	5.74	3	1.97
25	53	11.70	19	12.51
30	54	11.92	14	9.21
35	64	14.14	29	19.08
40	60	13.24	14	9.21
45	60	13.24	26	17.11
Total	453	100.00	152	100.00
Mean for 5-day period	50.3	11.11	16.8	11.11

No. fresh-flour eggs cf. No. conditioned, $P<.0000$

that fecundity is sensitive to the effects of environmental conditioning. Furthermore, these results cannot be attributed to altered egg-eating rates since it has already been shown that conditioned flour reduces cannibalism which would automatically favor maximum productivity by conditioned-flour cultures. In addition, the control and experimental data are too dissimilar to be explained by an altered rate of egg consumption; an actual difference in the number of eggs laid is the logical conclusion.

An examination of the percentage columns of Table II suggests an interesting possibility worthy of casual mention. These figures indi-

cate that there is a tendency for the control and experimental egg rates to vary rhythmically. In other words, when the control females are more productive, the experimental ones seem to be also; and when the controls are less productive, the experimentals are generally less. Since these suggestions arose incidentally out of an experiment designed to test another point, and since the number of cases (i.e., number of counts) is so small, it is inadvisable to draw any definite conclusions.

It might be worth while, however, to follow the matter a little farther to see if any trend in the variation can be biometrically shown. On the basis of chance alone it may be assumed that the expected number of eggs per 5-day count for both controls and experiments will be one-ninth of the total number of eggs produced (since there are nine counts). This would be one-ninth of $453 = 50.3$ for the fresh-flour series, and one-ninth of $152 = 16.8$ for the conditioned-flour series. The actual deviation of each of the nine experimental points from the expected, and each of the nine control points from the expected, may be calculated and a correlation coefficient obtained of these deviations. This was done, and it was found that

$$r = +.85 \pm .10 .$$

This is a rather high positive correlation coefficient which is more than eight times its probable error. This simply suggests that the control and experimental points (i.e., proportions) are associated so that there is a marked tendency for high egg production on the part of one to go hand in hand with high egg production on the part of the other.

The correlation between the rise and fall of successive counts of both control and experimental series was calculated. To illustrate, the fall in egg numbers between the first and second fresh-flour readings was obtained, and this figure paired with the fall shown by the first and second conditioned-flour readings. These figures were obtained for the total period and correlated. The coefficient was

$$r = +.72 \pm .18 .$$

This coefficient suggests that the two curves tend to follow similar trends, i.e., a rise from one count to the next is most frequently as-

sociated with a corresponding and simultaneous rise by the other series of data.

It should again be emphasized that the present data are not to be interpreted as demonstrating the existence of some factor, either extrinsic or intrinsic, which affects the rhythmicity of *Tribolium* reproduction. As before stated, the data on this point are incidental to the main problem and not numerous. It may well be that the whole matter has a purely coincidental explanation. On the other hand, the trends are suggestive enough to warrant notice. Chapman (1933), in an abstract, calls attention to experimentally demonstrated cycles of abundance and sparseness for *Tribolium* populations.

CONDITIONED FLOUR AND FECUNDATED-FEMALE FECUNDITY

Even though it can be demonstrated that conditioned flour reduces the fecundity of virgin females, it must be emphasized that this physiological fact is of no importance in population studies. The eggs produced by virgin females are infertile and consequently play no direct part in the population trend. Therefore, an experiment was set up to test the effect of conditioned flour upon fecundated *Tribolium* which compared oviposition rates of paired beetles in fresh and conditioned media. The control consisted of 20 bottles each containing 32 gm. of fresh flour and one pair of *Tribolium*. The experimental series was similar except that conditioned flour supplanted the fresh. The adult beetles used were isolated as pupae from the same stock with half of the group placed in fresh flour for 2 months and half placed in conditioned flour for 2 months. The males were kept apart from the females until the actual experiment was started. Egg counts were made daily of every bottle and the pairs returned to their respective environments. The eggs were isolated into shell vials (one vial for each bottle) and permitted to hatch so that the fertility rate could be obtained. This aspect will be discussed in the section on fertility. The experiment was run 11 days, permitting 11 daily egg counts to be made. It seems apparent that any change in egg numbers between control and experiment demonstrable in this investigation is due to an actual fecundity effect. It has been already pointed out that cannibalism is reduced by conditioning instead of accelerated, so that the difference observed is real since the controls

are more productive than the experimentals. Furthermore, it seems a safe deduction that cannibalism is a negligible factor in these experiments since all eggs were removed from the presence of the beetles every 24 hours. This enormously reduces the chance of a beetle meeting and consuming an egg. The data are summarized in Table III.

It is striking to note the difference in fecundity between the fresh-flour beetles and the conditioned flour ones. The former produced essentially ten times as many eggs as the latter, and the probability figure shows that there is no chance in ten thousand for such a result to be duplicated by chance. The coefficient of variability brings out the interesting fact that the conditioned-flour *Tribolium* were

TABLE III

FECUNDITY RATES OF FRESH AND CONDITIONED-FLOUR *Tribolium*

	Total No. Eggs Produced	Mean No. Eggs per Female for 11 Days	Standard Deviation	Coefficient of Variability (Per Cent)	Mean Rate per Female per Day	n
Fresh (*A*)	342	17.1 ± 1.01	6.7 ± .71	39. ± 4.16	1.5	20
Conditioned (*B*)	39	1.9 ± .40	2.7 ± .28	140. ± 17.15	.17	20

Conditioned cf. fresh, $P < .0000$

relatively much more variable than the controls. In fact, the experimental beetles laid eggs extremely erratically. This suggests that the organisms are fundamentally influenced by such conditioning.

Park (1934*a*) has shown that by returning conditioned-flour beetles to fresh flour their level of reproduction can be raised (in 40 days' time) to a point where it was essentially similar to the fresh-flour control. After the conclusion of the fecundity experiment described in the preceding paragraph, the adult *Tribolium* from fresh flour were placed as pairs into conditioned flour and the beetles from conditioned flour were introduced to fresh flour. These beetles remained in this changed environment for 2 months, whereupon their fecundity rates were again obtained. Thus the control consisted of 20 bottles, each containing 32 gm. of fresh flour, plus one pair of *Tribolium* which had lived $2\frac{1}{2}$ months in conditioned flour and 2

months in fresh flour. The experiment consisted of 20 bottles, each containing 32 gm. of conditioned flour, plus one pair of *Tribolium* which had lived $2\frac{1}{2}$ months in fresh flour and 2 months in conditioned flour. The bottles were incubated 11 days, egg counts being made at the end of that time. The data are presented in Table IV. In order to discuss the data more clearly, the fresh-flour beetles of the first experiment will be designated *A;* the conditioned-flour beetles of the first experiment, *B;* the conditioned-flour (originally *A*) beetles of the second experiment, *A′;* and the fresh-flour (originally *B*) beetles of the second experiment, *B′*.

TABLE IV

FECUNDITY RATES OF FRESH-FLOUR BEETLES ORIGINALLY IN CONDITIONED FLOUR, AND CONDITIONED-FLOUR BEETLES ORIGINALLY IN FRESH FLOUR

	Total No. Eggs Produced	Mean No. Eggs per Female for 11 Days	Standard Deviation	Coefficient of Variability (Per Cent)	Mean Rate per Female per Day	n
Conditioned A'....	231	11.5 ± 1.02	$6.8 \pm .72$	59.13 ± 6.30	1.04	20
Fresh B'.....	329	16.4 ± 1.53	10.2 ± 1.08	62.19 ± 6.63	1.49	20

Conditioned A' cf. fresh B'	$P = .0686$
Conditioned A' cf. conditioned B	$P < .0000$
Conditioned A' cf. fresh A	$P = .0070$
Fresh B' cf. conditioned B	$P < .0000$
Fresh B' cf. fresh A	$P = .8000$

The following conclusions may be drawn from the data of Tables III and IV:

1. Conditioned (A') and fresh (B') are similar with respect to fecundity although the probability of their difference is a borderline one. This suggests that the greater fecundity of B' may be a real thing.

2. Conditioned A' dropped significantly (one-third) when compared with A (original fresh). This drop was probably due to conditioning plus aging.

3. Fresh B' rose significantly over conditioned V after introduction to fresh flour.

4. The original conditioned (*B*) had a significantly lower rate of fecundity than the later conditioned *A'*.

5. The original fresh (*A*) and the later fresh (*B'*) had essentially the same fecundity.

6. The general conclusion to be drawn seems to be that conditioning, at least up through several months' time and with the flour used here, does not irrevocably alter the fecundity of *Tribolium*.

THE EFFECT OF MALE CONDITIONING ON FECUNDITY

While the previous experiments bring out the relation between conditioning of pairs and fecundity, they do not give any indication as to a possible differential effect upon the sexes. In other words, are only the females affected by conditioned flour, or can the males be influenced in such a manner that the fecundity of the females may be altered? An experiment was designed to test this point. Eighty *Tribolium* pupae were divided up so that 20 males and 20 females could be isolated into conditioned flour and 20 males and 20 females into fresh flour. These beetles were kept for 2 months in their respective environments. After this time 80 bottles were prepared, each containing 32 gm. of fresh flour, and four matings of 20 bottles each made. The matings were as follows:

a) Fresh-flour male × fresh-flour female
b) Fresh-flour male × conditioned-flour female
c) Conditioned-flour male × fresh-flour female
d) Conditioned-flour male × conditioned-flour female

The experiment was run for 11 days, at which time a census of all the populations was taken. The data, along with statistical comparisons, are presented in Table V and graphically shown in Figure 1. A number of instructive conclusions can be drawn from these figures: first, most of the results are as anticipated, i.e., the fresh-flour females were always greatly more productive than the conditioned-flour females, and the two groups of conditioned-flour females oviposited similarly irrespective of the past history of the males with which they were mated. The unexpected result becomes apparent when the fresh-male × fresh-female series is compared with the conditioned-male × fresh-female series. Here the former group are ovipositing at a significantly higher rate than the latter. This would

suggest that the conditioning of the males has, in some way, reduced the fecundity of their female mates, and, although this is not as important as the direct effect of conditioning upon the females them-

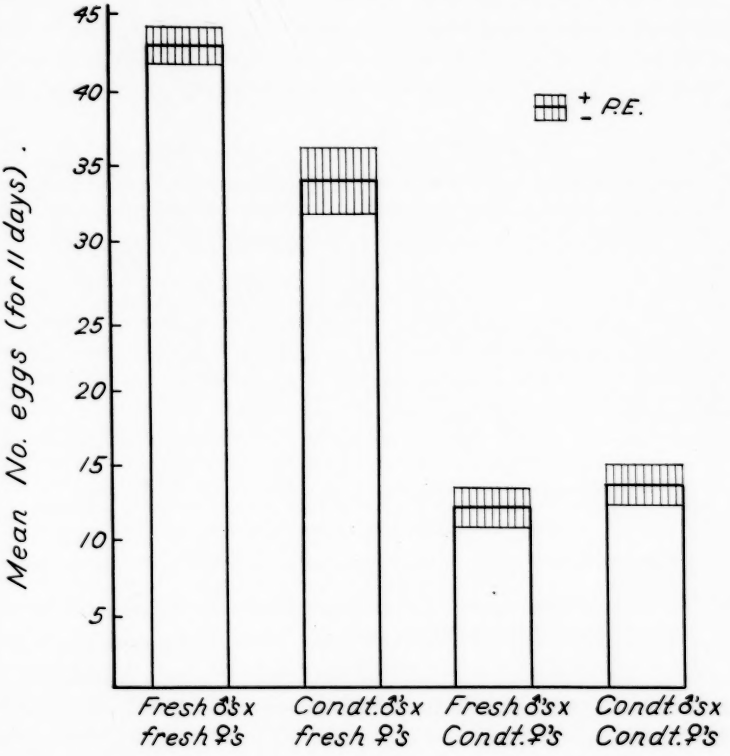

FIG. 1.—Productivity of beetles mated as follows: (1) fresh male × fresh female, (2) conditioned male × fresh female, (3) fresh male × conditioned female, (4) conditioned male × conditioned female.

selves, nevertheless it is a factor to be dealt with in thinking of population phenomena. Apparently, if females are conditioned, the presence of an unconditioned male is of no importance in increasing their fecundity. A conditioned male, however, does seem able to reduce

somewhat the fecundity of females that have spent all their life in fresh flour.

The coefficients of variability of Table V are quite interesting. They show clearly how productivity is associated with the type of medium. The fresh-fresh cultures have a reasonably low C.V., for such a biological variable as fecundity. When fresh-flour females were mated with males from conditioned-flour, the variation in egg

TABLE V

EGG COUNTS FOR *Tribolium* MATED: (1) CONDITIONED MALE×CONDITIONED FEMALE; (2) FRESH MALE×FRESH FEMALE; (3) FRESH MALE×CONDITIONED FEMALE; (4) CONDITIONED MALE×FRESH FEMALE

	Total No. Eggs Produced	Mean No. Eggs per Female for 11 Days	Standard Deviation	Coefficient of Variability (Per Cent)	n
Fresh ♂×conditioned ♀	245	12.2±1.28	8.5± .90	69.6±7.41	20
Conditioned ♂×fresh ♀	676	33.8±2.02	13.4±1.42	39.6±4.18	20
Fresh ♂×fresh ♀	862	43.1±1.17	7.8± .82	18.0±1.88	20
Conditioned ♂×conditioned ♀	277	13.8±1.44	9.6±1.01	69.5±7.35	20

Comparisons:* Condt. ♂-condt. ♀ cf. fresh ♂-fresh ♀ $P<.0000$
Condt. ♂-fresh ♀ cf. fresh ♂-condt. ♀ $P<.0000$
Condt. ♂-fresh ♀ cf. fresh ♂-fresh ♀ $P=.0126$
Condt. ♂-fresh ♀ cf. condt. ♂-condt. ♀ $P<.0000$
Fresh ♂-condt. ♀ cf. fresh ♂-fresh ♀ $P<.0000$
Fresh ♂-condt. ♀ cf. condt. ♂-condt. ♀ $P=.6172$

* Calculated by the method of "Student" (1925).

production increased relatively about half. The conditioned-flour females were much more erratic in their egg production than the latter (fresh-flour) group.

CONDITIONED FLOUR AND FERTILITY

Having shown that the fecundity of *Tribolium confusum* is drastically lowered by subjecting the beetles to conditioned flour, it becomes important to know whether the same thing holds for fertility. Previous indications on this question have been indefinite. Park (1934*a*) concluded tentatively from the data presented at that time that no clear-cut fertility effect was demonstrable. In the experiment discussed in the section on fecundity of this paper (Table III)

the eggs were isolated into vials after counting, and incubated so that the percentage of eggs hatching could be obtained. These results suggested that conditioning lowered the fertility rate since 69.5 per cent of the fresh-flour eggs hatched while only 23 per cent of the conditioned-flour eggs hatched. The latter figure has no statistical reliability, however, since the actual number of eggs isolated was so small in comparison to the number of fresh-flour control eggs isolated.

The problem was experimentally approached further. A control was set up containing 10 males and 10 females in 100 gm. of fresh flour. The beetles had spent 1 month in fresh flour prior to the start of the investigation. The experiment consisted of 10 males and 10

TABLE VI

EGG FERTILITY OF CONDITIONED AND UNCONDITIONED *Tribolium*

	Total No. Eggs Isolated	Total No. Eggs Hatched	Mean No. Eggs Hatched per Pair	Per Cent Fertility
Fresh-flour beetles.....	582	416	20.8	71.4
Conditioned-flour beetles....	582	382	19.1	65.6

Fresh cf. conditioned, $P = .2302$

females in 100 gm. of conditioned flour. These *Tribolium* were 1 month old at the start of the experiment and had lived since pupae in conditioned flour. Every 7 days the control and experimental bottles were sifted and the eggs collected. These eggs were placed in vials containing 10 gm. of fresh flour and allowed to hatch. Then the number of larvae were counted. The experiment was run in duplicate: there were two fresh-flour bottles of 10 pairs each and two conditioned-flour bottles of 10 pairs each. The results are recorded in Table VI. It can be seen that an appreciable number of cases have been collected and that no significant difference in fertility rate is apparent between the fresh- and conditioned-flour series. It would seem that fertility effects play little or no part in explaining the decline of *Tribolium* populations. Despite the clear-cut nature of these experiments, however, the possibility still remains that flour in other

stages of conditioning might have some influence upon fertility. The fertility percentages of Table VI are somewhat lower than those obtained by Park (1933), with a different experimental technique, or by Good (1933). These latter observations showed that normally about 90 per cent of the eggs hatch into larvae.

THE EFFECT OF CONDITIONED FLOUR ON METAMORPHOSIS

The importance of the relation of conditioned flour to metamorphosis is obvious in the study of population problems. Metamorphosis is a critical stage in the life-history of the beetle. If metamorphosis is considerably slowed down from normal, or if mortality is high during the period, the population as a whole suffers. Three aspects of this problem have been experimentally dealt with. These are: first, conditioned-flour effects on the egg stage; second, conditioned flour and the rate of metamorphosis; and, third, conditioned flour and the mortality of metamorphosis.

The question can be asked: Are the eggs affected in any way by simply being in conditioned flour during their period of incubation (5–7 days)? An answer to this question was sought experimentally. Twenty single matings were made of 4-month old, closely related male and female beetles. Each pair was placed in 32 gm. of fresh flour. Thus there were 20 of these environments. The flour was sifted daily and the eggs removed. The beetles were returned to their respective bottles. As the eggs from each pair were counted they were segregated into two equal groups. This was done for the eggs of all 20 pairs. Half of these eggs (one group) was placed into 10 gm. of fresh flour and half into 10 gm. of conditioned flour. This procedure was duplicated until 11 daily, consecutive segregations had been effected. The eggs were allowed to hatch in their respective bottles with larval counts made. In other words, eggs parentally similar are chosen at random and placed into fresh and conditioned flour to see if this variable has any effect on the success or failure of their development. The data are summarized in Table VII. It is at once clear that the conditioned flour does not affect the egg-stage mortality. This is, of course, the logically expected conclusion, there being no apparent reason why eggs merely present in conditioned flour should be adversely influenced.

An experiment was set up to study the rate and mortality of *Tribolium* metamorphosis in fresh and conditioned flour. About 300 eggs were collected from a small stock of closely related beetles and placed in a dish containing a slight quantity of flour. These eggs were allowed to hatch and 60 first-instar larvae, all within 12 hours of the same age, were collected. Thirty of these 60 larvae were used in the fresh-flour control and 30 in the conditioned-flour experiment. The control consisted of 30 vials, each containing 1 gm. of fresh flour and one of the randomly chosen larvae. The experimental vials were similar except for the fact that they contained conditioned instead of fresh flour. Every day each of these 60 vials were sifted and examined to see if an instar had been passed through. If the larvae

TABLE VII

EFFECT OF CONDITIONED AND FRESH FLOUR UPON THE EGG STAGE

	Total No. Eggs Segregated	Total No. Larvae Produced	Mean No. Larvae per Pair	Per Cent Fertility
Eggs segregated in fresh flour	133	85	4.2	63.9
Eggs segregated in conditioned flour	133	87	4.3	65.4

Larvae from fresh cf. larvae from conditioned, $P = .8808$

had molted (i.e., passed through an instar), the exuvia or shed larval skin was present in the medium. This criterion of molting was used by Good (1933), and was adopted here with the best of success. It seems to be a much more practical method of evaluating larval growth than that of measuring the head capsule as used by Bringley (1930). The presence of each exuvia was recorded according to time, date, instar, and individual case number. Larval deaths were also recorded. A number of larvae similar to the original 60 were isolated into control and experimental conditions, and run simultaneously with the actual investigation. These forms were used in replacing larvae that died in early metamorphosis. After the third instar, however, it was considered unwise to make any replacements since individual cases could not be adequately duplicated by the reserve larvae.

The data, with respect to the rate of metamorphosis of the control and the experiment, are biometrically presented in Table VIII, with a list of probabilities of differences in Table IX.

TABLE VIII

BIOMETRIC CONSTANTS FOR METAMORPHOSIS (TIME IN DAYS)

Stadium	Range		Mean Time	Standard Deviation	Coefficient of Variability (Per Cent)	n
	Maximum Time	Minimum Time				
	Fresh Flour					
i instar	3	2	2.1± .04	.33± .02	3.5± .31	30
ii instar	22	3	9.2± .51	4.1 ± .36	44.5±3.91	29
iii instar	12	4	5.8± .21	1.7 ± .14	29.0±2.56	29
iv instar	6	3	4.7± .08	.7 ± .05	14.8±1.28	29
v instar	6	5	4.8± .06	.48± .04	10.0± .87	29
vi instar	8	4	5.8± .10	.83± .07	14.3±1.28	28
vii instar	18	5	9.6± .43	3.4 ± .30	35.4±3.19	28
viii instar	15	6	9.5± .42	2.5 ± .29	26.3±3.13	16
ix instar	7	6	6.5	.....	.....	2
Pupal stage	10	6	7.5± .10	.81± .07	10.8± .97	28
Entire period	72	47	55.9± .72	5.7 ± .50	10.1± .91	28
	Conditioned Flour					
i instar	3	2	2.1± .03	.30± .02	4.2± .37	29
ii instar	28	2	14.5± .93	6.48± .65	44.6±6.75	22
iii instar	21	6	11.0± .49	3.8 ± .34	34.0±3.12	27
iv instar	27	6	12.7± .79	6.1 ± .55	48.0±4.4	27
v instar	37	6	13.7±1.01	7.8 ± .71	56.9±5.22	27
vi instar	19	5	10.3± .58	4.0 ± .41	38.8±5.21	21
vii instar	28	7	12.0± .93	5.7 ± .65	47.5±5.46	17
viii instar	20	9	11.8± .61	2.9 ± .43	24.5±3.69	10
ix instar	13	10	11.5	.....	.....	4
x instar	5	5	5	.....	.....	1
Pupal stage	13	5	8.6± .51	2.3 ± .36	26.7±4.24	9
Entire period	117	69	89.1±3.59	16.0 ±2.53	17.9±4.22	9

A number of interesting conclusions and suggestions may be drawn from these results as follows:

1. The conditioned-flour larvae took significantly longer (33 days more, or roughly one-third of the total time) to pass through their entire metamorphosis than did the fresh-flour larvae.

2. The relative variability in total time for the conditioned series is somewhat greater than for the fresh series although the difference is not statistically significant ($P = .2247$).

3. The coefficients of variability bring out several interesting points. In the first place, the individual instars varied in time, on the average, much more than did the entire period of metamorphosis. This is true for both conditioned and fresh series. This fact seems to suggest strongly that there is some compensation in rate of development, so that if a larva passes through one or several instars rapidly, it tends to have a longer than average instar sometime in the total

TABLE IX

PROBABILITIES OF DIFFERENCES BETWEEN TIME OF METAMORPHOSIS FOR STAGES IN CONDITIONED FLOUR AND STAGES IN FRESH FLOUR (DIFFERENCES BETWEEN MEANS)

Comparison	Probability
1st instar fresh cf. 1st instar conditioned	1.0000
2d instar fresh cf. 2d instar conditioned	.0007
3d instar fresh cf. 3d instar conditioned	< .0000
4th instar fresh cf. 4th instar conditioned	< .0000
5th instar fresh cf. 5th instar conditioned	< .0000
6th instar fresh cf. 6th instar conditioned	< .0000
7th instar fresh cf. 7th instar conditioned	.1208
8th instar fresh cf. 8th instar conditioned	.0365
Pupal stage fresh cf. pupal stage conditioned	.1378
Entire period fresh cf. entire period conditioned	< .0000

period. The C.V.'s are larger, in general, for the conditioned series than for the fresh; the fourth, fifth, and sixth instars of the conditioned showing much greater variability than the corresponding control stages.

4. The statistical comparisons of Table IX show the time of passing through the first instar to be similar for both control and experiment. Presumably the effect of conditioned flour has not made itself apparent at this time.

5. The fresh-flour second instar series pass through their development significantly faster than do the conditioned. This indicates a starting of the conditioning effect as early as the second instar.

6. The control, 3, 4, 5, and 6 instars are all significantly shorter than the experimentals.

7. From the seventh instar on the rate is more or less erratic.

8. The length of the pupal periods for both fresh and conditioned is statistically similar. The variability of the latter, however, is significantly more ($P = .0152$) than of the former.

9. These data confirm the opinion of Good (1933) that 7 or 8 instars are more typical for *Tribolium* than the usually postulated 6. It may be, however, that under more optimal conditions (possibly using a richer food) 6 instars would be experienced more frequently.

A brief statement may be made about the mortality associated with the fresh and conditioned-flour larvae. Of the 30 fresh-flour larvae there were two deaths: one in the second instar and one in the fourth instar. These two control deaths were not replaced by new larvae. Of the conditioned-flour larvae there were 41 deaths in all. The fact that more larvae died than the original number present (30) means that replacements were made from the reserve conditioned group. These replacements were all made during the first, second, and third instars since the stages could not be properly duplicated after that time. In some cases there were several replacements for the same vial. Nine of the larvae passed through the pupal stage and emerged as adult beetles. Three of these nine were of the original group. The highest larval mortality occurred during the second instar. These results are clear cut enough, without further analysis, to bring out the point that conditioning greatly increases the mortality of metamorphosis as well as slowing down the rate of development. Therefore, the physiological effect of conditioned flour upon metamorphosis becomes of great importance in helping to explain *Tribolium* population decline in heavily conditioned flour.

DISCUSSION AND CONCLUSIONS

The attempt has been made in this paper to analyze some of the physiological effects of self-contaminated conditioned flour upon the flour beetle *Tribolium confusum* Duval and its populations. Conditioned flour may be looked upon, in a sense, as a "population product." That is, it represents an environment which has been modified, as far as is known, through the activity of the beetles themselves. The flour, at least within limits, becomes more and more altered or conditioned as it is inhabited by the beetles. Since populations of

Tribolium eventually decline, and since this production of conditioned flour goes in step with the decline, it has seemed important to analyze the effects of heavily conditioned flour upon certain physiological processes of *Tribolium* to see the relation, if any, of this self-altered environment to the decline of the cultures. This is a logically necessary step in the proof of the reality of conditioned-flour phenomena. In a past paper (Park, 1934*a*) it was experimentally shown that early populations attained a much smaller size if the beetles were kept in conditioned flour than they did if the beetles were living in fresh flour. In the present paper the physiological processes, rate of egg-eating, strength of fecundity and fertility, and rate and mortality of metamorphosis have been experimentally dealt with, relative to the influence of conditioned flour upon them. These processes are all of great importance in altering and integrating population trends. For example, a changed rate of egg-eating could favor or disfavor the population as a whole; an increased fecundity or fertility rate would make for larger cultures; a slowing-up of metamorphosis associated with an increase of mortality could adversely influence the population. It has been here demonstrated that some of these processes are highly sensitive to the presence of conditioned flour. The results strongly point to the conclusion that the effects of conditioning upon the beetles are very influential in bringing about the decline of their populations. It is emphasized here that little knowledge is as yet available concerning the actual potent factors which produce the demonstrated effects. As Park (1934*a*) has previously pointed out, conditioned flour may simply represent an environment from which food material has been largely extracted by beetle action, or (and) an environment toxically charged with various *Tribolium* waste products. It may be that the flour produces its effects by infecting the *Tribolium* with a sporozoan parasite such as *Adelina* (Park, 1934*b*), thus altering and impairing their normal functions. Any or all of these possibilities, as well as others more subtle, can be tentatively thought of as the dynamic agents involved. Further investigations may bring more facts to light. The present data permit the following two conclusions: first, heavily conditioned flour favors decline of *Tribolium confusum* populations; and, second, the decline is probably primarily induced by reducing the fecundity of adult

beetles, lengthening the time of metamorphosis, and increasing the mortality of metamorphosis. Data as to the effect of conditioned flour upon longevity, also an important factor, are not available.

SUMMARY

1. Conditioned flour reduces the cannibalism or egg-eating of *Tribolium* about half that of the control rate of beetles in unconditioned flour.

2. Conditioned flour reduces the fecundity of virgin females about two-thirds that of controls. Growing out of this experiment is a suggestion as to the possible existence of a reproductive rhythm of egg production for the flour beetle.

3. Conditioned flour reduces greatly the fecundity of fecundated females mated with one male. This is not an irreversible effect as the conditioned forms may be returned to control level of reproduction by reintroduction to fresh flour.

4. A male from conditioned flour can lower the fecundity of females from fresh flour when mated to the latter. On the other hand, fresh-flour males cannot raise the fecundity of conditioned-flour females.

5. No definite effect of conditioned flour on fertility has been demonstrated. However, the possibility of the existence of such an effect is not denied.

6. Conditioned flour definitely increases the time and variability of larval metamorphosis.

7. Conditioned flour drastically increases the mortality of larval metamorphosis.

8. All of the foregoing remarks apply to the effects of heavily conditioned flour only.

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THE RÔLE OF STARVATION IN CONJUGATION OF PARAMECIUM

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MAUPAS (1889) observed that the addition of a suitable nutrient to a culture of *Paramecium* (or other ciliates) resulted in a rapid multiplication of the animals followed by their waning vigor and conjugation. Such a cycle was repeated subsequently to a second addition of hay or other nutrient following conjugation. However, conjugation was temporarily inhibited when the nutrient was added just before the animals had begun to conjugate. He therefore concluded that following a period of asexual divisions there came a period of karyogamic maturity, at which time conjugation would occur if the animals remained in a medium depleted of food.

Jennings (1910) states that, since in his studies *Paramecium* (*aurelia?*) conjugated only after a period of plentiful food (addition of hay), it is really "hunger after satiety" which induces conjugation, not starvation per se. He also observed conjugation in one case after as few as four asexual divisions.

Zweibaum (1912) found that even when vigorous *Paramecia* (*caudatum*) are starved following feeding they will not conjugate unless a particular salt environment is provided. Diluting with $FeCl_3$ and several other salt solutions he obtained excellent epidemics of conjugation. Ball (1925) repeating these experiments with *Paramecium caudatum* obtained negative results as had Hopkins and Jollos before him.

Hance (1917) using a large *Paramecium* with extra contractile vacuoles (probably *P. multimicronucleata*, see Landis, 1925) observed that, "Conjugation appeared in practically all cases when the culture was evaporated rapidly causing the metabolic products to concentrate rapidly." Chatton and Chatton (1931) found that *P. caudatum* will conjugate only in the presence of a particular bacterium (either in pure or in mixed culture). They concluded that

this bacterium produces a zygogenic metabolite essential for conjugation.

There is therefore considerable difference of opinion as to the factors inducing conjugation of *Paramecium*. Practically all authors observed that what appeared to be starvation and conjugation were parallel in their cultures. However, no experimental verification of the causative nature of hunger is available, since at the time depletion of food occurs metabolites of both Paramecia and bacteria have accumulated, and many other changes have occurred in the medium (cf. pH). Similarly, when nutrients are added to an exhausted infusion not only are the food bacteria enabled to increase but many other conditions of the medium are radically modified.

An attempt was made in this laboratory to induce epidemics of high percentages of conjugants of *Paramecium* under a repeatable set of conditions and then to study some of the factors necessary for conjugation under conditions as closely controlled as possible.

EXPERIMENTAL

The Paramecia (*Paramecium multimicronucleata*, Powers and Mitchell) were grown on a single strain of bacteria, *Pseudomonas ovalis*, in the manner described in another paper (Giese and Taylor, 1935). Briefly, they were cultured at 26° C. in 0.05 per cent lettuce extract buffered at a pH of 7.0. In such a culture a few conjugants normally appeared on the fourth to the fifth days. Various factors were varied to induce greater percentages of conjugation—namely, the concentration of the substrate, the exposure to the air, the amount of buffer, and the temperature. Success was attained when the standard cultures of *Paramecium*, $3\frac{1}{4}$–$3\frac{1}{2}$ days since the addition of Paramecia, were decanted on sterile Syracuse watch glasses held in sterile Petri dishes and were placed in an incubator at 19°–21° C. Therefore, in succeeding experiments, this method was followed.

At the time the culture is $3\frac{1}{4}$–$3\frac{1}{2}$ days old there are about 250 to 300 Paramecia per cc. They have for the most part ceased dividing and are becoming transparent and thin; food vacuoles are few or absent. After transferring to the watch glasses, the Paramecia settle to the bottom. In about 6–12 hours the sticky state (Calkins and Cull, 1907) appears, and the Paramecia aggregate in groups with some-

times as many as twenty in a mass. Then they gradually pair off, until after about six more hours the greater part of the Paramecia are conjugating. Some of the conjugants stained with iron hematoxylon showed disintegrating macronuclei, others, dividing micronuclei. We are therefore dealing with true conjugation, not mere adhesion.

The results of a typical set of experiments are given in Table I. The estimates were obtained by averaging the numbers of Paramecia

TABLE I

INCIDENCE AND INTENSITY OF CONJUGATION
(IN PERCENTAGE OF CONJUGANTS)

Hours after Transfer	Exp. 349							
	A	B	C	D	E	F	G	H
3	0	S*	S	S	S	S	S	S
6	50	25	50	25	75	75	0	0
12	75+	75	50	75	75+	75+	75−	75−
17	75	75	75	75+	75	75	50	75−
22	75	5	25−	75+	50	25	50	75
26	1	1	5	75	50	25	25−	50
29	0	0	1	5	5	1	1	5
43	0	0	0	1	0	1	0	0

* S = sticky.

conjugating and not conjugating in several regions of the container. Sample counts were not made, since they would expose the culture to infection as well as render impossible a series of readings on the same set of cultures.

The onset of conjugation seems to follow the exhaustion of the food supply. Is the absence of food a necessary condition or is it merely coincident with other conditions leading to conjugation? This might be tested by adding bacteria as food without markedly changing the medium in any other way. A loopful of a 24-hour slant of bacteria was accordingly added to a series of $3\frac{1}{2}$-day cultures in watch glasses. Table II illustrates the results obtained. These cultures represent one-half portions of the culture fluid of the same set of cultures given in Table I; the experiments of Table I are therefore controls to the experiments of Table II. Following the addition of bacteria, the Paramecia gorged, became large, and in most cases di-

vided. Then as they used up the food, they became free of food vacuoles and gradually began to conjugate. It is clear that feeding delays conjugation, and that while food is present conjugation does not occur even though the medium has remained relatively unchanged.

Starving ex-conjugants dwindle away without reconjugating. When fed again and then starved, however, they again conjugate.

TABLE II

DELAY OF CONJUGATION BY FEEDING (IN PERCENTAGE OF CONJUGANTS)

Hours after Transfer	Exp. 349						
	A	B	C	D	E	G	H
6	D*	D	D	D	D	D	D
12	0	0	0	0	5	S	D
17	0	0	0	0	25	S	0
22	0	0	0	0	25−	S	0
26	0	0	1	0	5	5	0
29	0	0	25	S	50	25−	0
43	75+	75+	75+	75	75+	50	75+
48	75	75−	25−	75−	75−	25−	75+
52	75−	50	5	25	5	1	75
64	0	0	0	0	1	0	5

* D = dividing.

Such a set of experiments is summarized in Table III. As will be observed from this table, the first conjugation epidemic occurred in from 8 to 18 hours after transferring to the watch glasses. In 66 hours after transferring a small loopful of bacteria sufficient to enable the Paramecia to regain a healthy condition was added and for 12 hours the cultures were placed at 26° C. to accelerate the exhaustion of the added food. They were then returned to the chamber at about 20° C. In 13–26 hours the second epidemic occurred (Table III). Controls showed no conjugation at a corresponding time. No cytological studies were made of the Paramecia in the two successive epidemics of conjugation.

It appears as though lack of food after the Paramecia had been well fed leads to a particular chemophysical state of the protoplasm resulting in the sticky state, pairing and conjugation (In all the above

experiments the usual precautions were observed to prevent the infection of cultures with other strains of bacteria and test streaks were made for all of the experiments reported.)

Is the onset of conjugation entirely independent of all the other factors of the medium? At the time of conjugation the medium contains metabolites of the Paramecia and the bacteria, modified constituents of the lettuce extract, and salts from the lettuce extract as when Paramecia are washed free of these materials the Paramecia

TABLE III

RECONJUGATION AFTER FEEDING (IN PERCENTAGE OF CONJUGANTS)

Hours after Transfer	Exp. 353							
	A	B	C	D	E	F	G	H
3	S	S	0	5	S	S	0	S
8	75+	75−	75+	75	25	50	S	50
18	75+	75+	75+	5	75	75	75	25−
22	75−	75	50	1	75	50	75−	5
26	5	75	<25−	0	5	50	75−	5
31	0	5	1	0	5	1	1	1
43	0	0	0	0	1	0	0	0
66	fed	fed	fed	fed	fed	fed	fed	fed
91	25−	S	75+	25	5	1	1	5
94	25	50	75+	50	75+	50+	25	50
99	75−	50	75+	75	75+	50	50	50
104	75−	50	50	25	75+	75	50	50
116	0	1	0	0	0	1	1	0

well as from the buffer. To determine whether conjugation will occur were treated in the following manner. A 7 cc. portion of the culture was centrifuged lightly at about 10 gravities for 1 minute, the supernatant fluid was withdrawn, leaving the Paramecia in from $\frac{1}{4}$ to $\frac{1}{2}$ cc. of culture medium. Doubly distilled water was added, and after shaking, the centrifuging was repeated. After withdrawing the supernatant fluid the procedure was again repeated, following which the Paramecia were suspended in 7 cc. doubly distilled water. (Previous work had shown that starving Paramecia can live in doubly distilled water for 10 days appearing entirely normal.) Conjugation occurred to only a slight extent (1 per cent) or not at all in the cultures so washed at the time when the controls exhibited a normal epidemic. Doubly distilled water is therefore not a favorable medium for con-

jugation. (That centrifuging is not deleterious in the above experiments is shown by the fact that Paramecia, centrifuged in culture medium from 1 to 5 minutes, conjugated comparably to controls which had not been centrifuged.)

To test whether conjugation will occur when the Paramecia are washed and suspended in balanced salt solutions the Paramecia were now washed four times in diluted Osterhout's balanced salt solution buffered at a pH of 7.0 with 1 cc. of the standard buffer per 10 cc. salt solution (Barker and Taylor, 1931), using in general the method outlined for distilled water. The controls were Paramecia in culture medium. In the first six experiments counts of the numbers of conjugants and non-conjugants in 1 cc. samples (after mixing) were made; in the last three experiments the entire cultures were counted. In all except the first two experiments the number of Paramecia per given volume of fluid was doubled by centrifuging and withdrawing one-half of the fluid before transferring to watch glasses. The mean for the experimental cultures was found to be 69 ± 1.9, the range 60–85; the mean for the controls was 67 ± 2.5, the range 45–85. The conjugation in the salt solutions is therefore entirely comparable to that in the controls.

In the foregoing experiments the bacterial metabolites have been diluted some 100,000-fold, yet conjugation occurs just as in the controls. Apparently within these limits conjugation is independent of the concentration of bacterial metabolites. While it is possible that these metabolites are zygogenetically active even at such high dilutions, and that there is no relation of conjugation to their concentrations, it is rather improbable. The "azygogenic" bacteria of Chatton and Chatton (1931) may have produced substances which inhibit conjugation. If this is true, washing the Paramecia free of these toxic agents should enable them to conjugate. Paramecia are now being grown in this laboratory on several strains of bacteria in an attempt to find which strains are zygogenic and which azygogenic in order to test the above suggestion.

The conjugation of *Paramecium* seems, also, to be independent of the concentration of its own metabolites within certain limits. For example, Paramecia washed in the culture medium of an old stand-

ard 0.05 per cent lettuce culture or a 0.1 per cent lettuce culture in which the inhabitants have for the most part died out (2–4 weeks) and in which metabolites are probably in considerably higher concentration than in controls, conjugate comparably to controls. Also, the Paramecia held in salt solutions conjugate similarly to controls (Table IV) even though the metabolites have been diluted. Finally the few Paramecia present in a mixed Protozoan culture in which Paramecia had never been numerous were found in conjugation. Considering their numbers and the volume of the culture, their metabolites must have been present in insignificant concentration.

It appears that conjugation will occur over a wide range of concentration of metabolites of both bacteria and Paramecia. There is, however, always a correlation of conjugation with the lack of food (after a period of sufficient food).

As to the mechanism of induction of conjugation by starvation, little evidence is as yet available (Zweibaum, 1922). It is desirable to accumulate more data on the effects of some of the other variables upon conjugation before attempting this difficult though fascinating problem.

DISCUSSION

These experimental results are in accord with many of the observations on conjugation not only of *Paramecium* as already noted, but of many other Protozoans. Thus Popoff (1908) cites starvation as attendant to conjugation in *Carchesium polypium*. Collin (1909), studying the conjugation of the parasite, *Anoplophyra branchiarum*, believes that lack of food may be one of the main conditions attending the epidemic of conjugation, since conjugation occurs when the number of parasites has reached a maximum. Mast (1917) observed that lack of food in addition to crowding and a favorable temperature is an important factor in inducing conjugation in *Didinium nasutum*. Calkins (1919) found that *Uroleptus mobilis* will conjugate when starved, provided the internal conditions are suitable. Austin (1927), however, obtained contradictory results as to the causative nature of hunger for the same animal. Woodruff and Spencer (1924) found that the carnivore, *Spathidium spathula*, can be induced to conjugate by "allowing the animals to remain for a day or two without an adequate supply of food." Bělař (1924) found that *Actino-*

phrys sol will conjugate in acid as well as in alkaline media, in a medium previously occupied by *Actinophrys* as well as in fresh media, provided food is lacking. Adding food (*Gonium pectorale*) in any of the above cases prevents conjugation as long as the food supply lasts. Conjugation occurs as rapidly in a crowded culture as in a sparse one, when the food is exhausted. He concludes that lack of food is an essential factor for inducing conjugation.

Conjugation seems to be readily inhibited. Temperatures of 26°C. or above were found to be deleterious to conjugation in the present reported experiments. What seems to be excessive accumulation of metabolites, following many feedings of bacteria to Paramecia in a watch glass, results in such injury to the Paramecia that they are unable to conjugate even when the food supply is exhausted. Similar results are recorded by Bělař for *Actinophrys*. High concentration of substrate is unfavorable to conjugation possibly because there is so gradual a diminution of available food that the critical state is not reached by many Paramecia at the same time. Paramecia suspended in salt solutions are readily injured by slight traces of toxic agents so that very great care had to be taken in these experiments. A more complete study of the inhibitory phase of the problem is now being undertaken.

The variation in the conjugating capacity of different clones has been repeatedly noted and Calkins (1913) calls attention to such variation even among the progeny of a single clone. The clone used in the present experiments showed the same capacity to conjugate at various times throughout the year and a subclone recently isolated was similar to the parental clone in this respect. Studies on other clones are planned in the next series of experiments.

SUMMARY

A method for inducing conjugation in a clone of *Paramecium multimicronucleata* is outlined; conjugation follows the exhaustion of the bacterial food.

Addition of bacteria to an exhausted culture just about to conjugate delays conjugation; following exhaustion of this food, conjugation occurs.

While Paramecia fail to conjugate after being washed in doubly

distilled water, they conjugate comparably to unwashed controls when washed in balanced salt solutions, even when the culture medium has been diluted some 100,000-fold.

In all cases conjugation is parallel to the exhaustion of food, and independent, within the limits studied, of the concentration of metabolites of the Paramecia or the bacteria.

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